The role of Microbe-Matrix interactions in dairy starter culture functionality

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The cover shows the 3D milk matrix fermented with *Lactococcus lactis* B40. The image was taken by Jan Klok/M.Tarazanova with confocal laser scanning microscopy (zoom 4, 13 µm deep into the sample) by merging 3 pictures of z-scan. The Argon laser was used to visualize the Syto 9-stained bacteria, while the DPSS 561 laser was used to visualize the milk protein and fat droplet in the matrix that were stained by the Acridine Orange and Rhodamine B, respectively. The objective lens used was an HCX PL APO 63×/1.2 /water CORR CS. Bacterial cells are green, proteins and lipids appear orange/red; the black areas represent the serum fraction. For other examples of fermented milk microstructure please see Chapter 4.

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Contents

Chapter 1

General introduction

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Microbe – Matrix interactions in fermentation processes

Abstract

Microorganisms play an important role in industrial processes such as food- and feed-fermentation, the production of biofuels, bio-pharmaceuticals, wastewater treatment and bioremediation. For the optimization of such a variety of processes several common approaches can be employed, including the exploitation of biodiversity, the adaptation of organisms to particular environments, and the detailed characterization and subsequent engineering of desired phenotypes. In general these methods focus on microbial growth in combination with particular enzyme activities that are desired in an industrial process. Although there is ample literature on microbial surface properties and how these influence interactions of the microorganisms with their environment, there seems to be little information on the role of such interactions in an industrial setting. Here we review if and how microbial surface properties can influence industrial food fermentation processes. We will restrict ourselves to the lactic acid bacteria (LAB) and examine whether there might be hitherto unused potential to improve fermentation processes using these versatile microorganisms, which play a major role in the production of healthy and nutritious foods for humans worldwide.

Introduction

Bacteria show considerable differences in the composition and structure of their cell walls. One of the oldest and best known methods detecting differences in cell walls is a staining procedure developed by Danish bacteriologist Hans Christian Gram in 1884 (1). Gram staining is based on the observation that bacterial cells with a cell wall containing a thick peptidoglycan (PG) layer retain crystal violet-iodine (CVI) complexes and stain purple; these bacteria are called Gram-positive. Conversely, Gram-negative cells have a thin cell wall of a few layers of PG surrounded by an outer membrane with hydrophobic layer of lipopolysaccharides (LPS) and lipoproteins (LP) (Fig. 1). These readily lose the CVI complexes upon alcohol treatment because the outer membrane dissolves and pores in the thin PG layer are big enough for CVI complexes to escape. Addition of the dye safarin colors Gram-negative cells pink (2). Because of their thick PG layer Gram-positive bacteria are generally more robust against physical stress than Gram-negative bacteria (2). Major differences between Gram-positive and Gram-negative bacteria are given in Table 1 (for more detailed information see (3)).

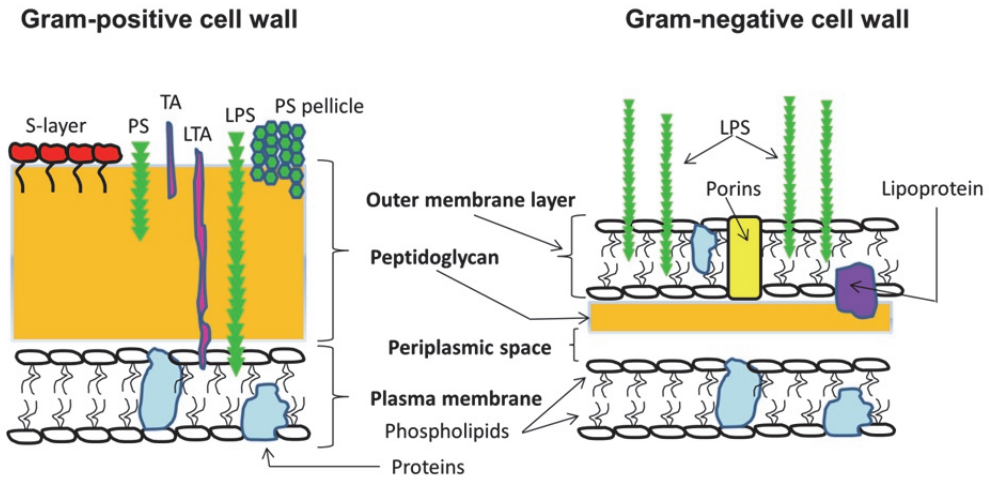

Figure 1. Cell wall structure of Gram-positive and Gram-negative bacteria. PS stands for polysaccharides, LPS – lipopolysaccharides, TA – teichoic acid, LTA – lipoteichoic acid. The cytoplasmic membrane consists of phospholipids and proteins and forms a permeable barrier responsible for transport of solutes and energy generation.

Short and thin (1-10 nm in diameter) hair-like projections called fimbriae or pili are anchored in the cell wall of both Gram-negative and Gram-positive bacteria (4). Pili consist of pilin proteins; these are classified in several types based on the structure assembling, functionality, and occurrence in bacterial species (4). For example, type I pili are found mostly in *E. coli*; they have adhesive properties, allowing the bacteria to attach to other bacteria or to surfaces. Type IV pili generate motile forces and are mostly present in Gram-negative bacteria and in two Gram-positive species. When pili attach to other bacteria or to a surface, they contract and pull the pilin-producing bacteria forward. Such motility is jerky and differs from motility generated by flagella. Moreover, both Gram-negative and Gram-positive species can exchange genetic information, mainly in the form of plasmids, through a cell-to-cell contact called conjugation and involving pilin-like structures (5–7). Flagella are lash-like appendages made of the protein flagellin that protrude from the cell wall. Flagella are longer and thicker than pili. Their main function is in locomotion and they allow flagellated bacteria to glide on slime they secrete. Flagella have a sensing function in some bacteria

allowing them to respond to environmental conditions (8). More information about flagella and flagellar organization and function is given in references (9–11).

In natural environments like biofilms, in order to protect bacteria, outside the cell wall of both Gram-negative and Gram-positive bacteria a layer called glycocalyx can be found (12). Glycocalyx is a network of polysaccharides. When cells grow planctonically, a glycocalyx layer is usually absent. More information on the glycocalyx in Gram-positive bacteria is given below.

Table 1. Some cell wall differences between Gram-negative and Gram-positive bacteria. PG stands for peptidoglycan, LPS – lipopolysaccharides, LP- lipoproteins, TA – teichoic acid, LTA – lipoteichoic acid.

Cell wall composition	Gram-positive bacteria	Gram-negative bacteria	References
PG layer	20-100 nm	2-7 nm	(3, 13, 14)
Attachments to PG	TA, LTA, polysaccharides	-	(14)
Outer membrane	-	7-8 nm, LPS, LP	(15)
Motility	Non-motile, rarely motile	Motile or non-motile	(8)
Appendages	Pili	Pili, fimbriae	(4, 16–18)
Other components	Capsules	Capsules, lose slime	(19, 20)

Cell wall structure of lactic acid bacteria

Lactic acid bacteria (LAB) are Gram-positive bacteria that are used throughout the world for the production of fermented milk, meat, fish and vegetables products for human consumption, as well as for the fabrication of animal feed. Some LAB are (opportunistic) pathogens in humans and other animals. The genera of LAB comprise *Lactococcus, Leuconostoc, Lactobacillus, Streptococcus, Pediococcus, Aerococcus, Carnobacterium, Enterococcus, Sporolactobacillus, Oenococcus* (21). Their cell walls are typically some 50 nm thick and are, in some species, covered by an outer coating of proteins that form a so-called S-layer (Fig. 1).

Peptidoglycan

PG is a polymer of N-acetyl-glucosamine-β (1→4)-N-acetyl-muramic acid and its structure provides cells with strength to withstand turgor, supports their shape and protects them from environmental factors such as mechanical damage, antibiotics or enzymatic and osmotic lysis (16, 22, 23). The PG is also involved in cell division and growth, and behaves as a sieve, restricting secretion of proteins larger than 25 - 55 kDa for globular proteins (13, 14). Depending on the LAB species, the PG layer can be decorated with various molecules like teichoic acids (TA), lipoteichoic acids (LTA), polysaccharides, teichuronic acids (TUA), lipoglycans (lipopolysaccharides (LPS)) and lipoproteins (Fig. 1). For many LAB to the end of the D-lactyl carboxyl groups of N-acetyl-muramic acid five amino acids are attached: L-alanine, D-Glutamic acid, L-lysine, and two molecules of D-alanine. Reduction in D-alanine substitution of LTA increases LTA attachment to magnesium ions (14). For several lactobacilli it was observed that if one D-alanine residue is substituted by D-lactate or D-serine, the bacteria become resistant to vancomycin, a glycopeptide antibiotic (14). In *Lactococcus lactis* the decoration of LTA with galactose rendered the bacteria resistant to bacteriophage attack (24, 25), and reduced D-alanylation of LTA increased lactococcal cell lysis (26). In *S. aureus* the amount of D-alanine residues in LTA negatively correlates with anti-autolysin activity (27).

Teichoic acids (TA) are anionic polymers consisting of poly-glycerol phosphate, poly-ribitol phosphate, or poly-glycerol phosphate. For example, the *Lactobacillus plantarum* cell wall contains poly-(ribitol phosphate) TA. TA are formed on the outer side of the cytoplasmic membrane. TA and TUA are covalently bound to the PG, whereas LTA and lipoglycans (LG) remain attached to the cytoplasmic membrane, although a fraction of the latter may be found in a free form in the cell wall or released in the medium (14).

Cell walls carrying TA with poly-glycerol- or poly-ribitol-phosphate units are negatively charged. Similar to TA, TUA, a phosphate-free acid, is also negatively charged due to the carboxyl groups of glucuronic acid. In *B. subtilis* several genes have been identified:

tagD encodes a glycerol-3-phosphate cytidylyltransferase (an enzyme responsible for the TA synthesis) (28), *tagGH* encoding a two-component ABC transporter responsible for the TA translocation through the membrane (29), and an 8-cistron *tuaABCDEFGH* operon containing the genes necessary for TUA biosynthesis (30). Some genes related to TA and TUA biosynthesis in LAB have also been described. For example, *tagH* of *L. lactis* MG1363 is involved in export of TA (31). In *L. lactis* IL1403 a cluster of 7 genes *tagBD1D2FLXYZ* (≈ 950 kb) were reported and only 3 genes from pathway of TUA biosynthesis were found: *ycbK*, *ycbF*, and *ycbH*, corresponding to *tuaBCG* of *B. subtilis* (32).

Another PG-decorating compound is the S-layer, which among the LAB has only been found in the genus *Lactobacillus* (14, 33, 34). The S-layer consists of proteins (SLPs) that can be glycosylated (14). This layer non-covalently connects to PG via so-called surface layer homology (SLH) domains. If SLH domains are absent the connection occurs via electrostatic interactions between the positively charged N-terminus of the SLP and a negatively charged secondary cell wall polymer (e.g. TA). The exact role of S-layers is unknown, although adhesion, exclusion of harmful hydrolases, sieving (mainly retention) of large molecules, and masking of phage receptor have all been suggested as possible functions (35, 36). Despite the fact that SLPs have a pI above 9 (37), the isoelectric point of bacteria may differ from that of the SLPs because of other surface components located below the layer of SLPs (37).

Polysaccharides

Polysaccharides forming a glycocalyx can be divided into three groups, i) capsular polysaccharides (CPS), ii) wall polysaccharides (WPS) and iii) extracellular polysaccharides (EPS) (20). CPS form a thick layer around the cell and are covalently anchored to the PG. The capsule layer usually consists of different polysaccharides, depending on the type of bacteria (19), and can bind up to 99% of water (38). The polysaccharide capsule can protect the bacterial cell from phagocytosis but also from desiccation and facilitates the adherence to surfaces. The structure of CPS is species- or even strain-dependent and one strain may have different polysaccharides at the same

time. The composition of CPS depends on growth conditions (39). For example, *Lactobacillus casei* subsp. *rhamnosus* NCTC 6375 forms two polysaccharides, a hexosamine-containing H-polysaccharide (20%) and a rhamnose-containing R-polysaccharide (44%) when it is grown in media with glucose. The R-polysaccharide concentration decreases by approximately 50% when the cells grow in a medium containing fructose. A similar decrease in R-polysaccharide was obtained when cells were grown with rhamnose or ribose (39). CPS are considered to be virulence factor of pathogenic microbes and not perceived as a common feature in food bacteria such as *L. lactis*. However, CPS were found to form a sugar pellicle on the outer cell surface of *L. lactis* strains MG1363 and IL1403 used for food fermentations (20). Like the CPS, the sugar pellicle polysaccharides are covalently linked to PG, but in contrast to CPS, they form a thin layer. Sugar pellicle polysaccharides consist of repeating units of hexasaccharides connected by phosphodiester bonds. Such a structure is similar to that of TA or LTA but it does not contain glycerol, ribitol, or glucitol (20).

In contrast to the capsular layer, the slime layer around certain bacteria consists of glycoproteins and glycolipids in addition to polysaccharides (36, 40). This slime layer is far more unorganized in structure and can be easily removed (41). The slime layer protects bacteria from detrimental environmental components and conditions like antibiotics and desiccation and helps in bacterial surface attachment (42). Polysaccharides forming the slime layer might be EPS as well as WPS. EPS are not attached to the bacterial cell wall while WPS are covalently linked; however, WPS can be not attached to the bacterial cell wall and in this case WPS do form a capsule. Genes encoding the enzymes responsible for EPS formation, chain polymerization and export have been identified in *L. lactis* B40. The gene cluster is located on a plasmid and consist of the 14 genes *epsRXABCDEFGHIJKL* (43, 44). The major EPS components are hexose, pentose, hexosamine and ketose (45). Certain EPS can be attached to PG without covalent bonding (14). Sugars in EPS increase water absorption ability and, thus, influence the hydrophilicity of the bacterial cells. The biological function of EPS is not well understood but it is thought to be important for immune evasion of

pathogens, for biofilm formation (36), gliding, protection against osmotic stress, desiccation, binding of metals and horizontal gene transfer (46, 47).

Polysaccharides can be involved in cell precipitation or cell agglutination when they are bound by proteins called lectins (36, 48). Thus, lectins inhibit bacterial attachment to surfaces or a substance by occupying the active sites responsible for such attachment (49–51). From a technological point of view EPS is important during dairy product manufacturing as it plays a key role in the formation of texture (14), in thickening, as a suspending agent, or as an emulsifying or cation-chelating compound (44, 52, 53).

Based on the information presented above, it can be concluded that the physicochemical surface properties of LAB are determined by a multitude of factors. Each of these separately or in different combinations could be important in the interaction between bacterial cells and (food) matrix components such as solid surfaces or particles in the sometimes complex fermentation media. Examples of such particles are proteins (e.g., in casein micelles), fat (droplets), polysaccharides, or fibers in various fermented products.

Cell morphology and chaining

LAB have either coccoid or rod like cell shapes and vary in size from a little under 1 μm to over 9 μm in length (54). They grow as single cells or in short or longer chains depending on growth conditions. Planktonically grown cells can aggregate or clump. Autolysins are PG-degrading enzymes that are very important for normal cell growth and division. The deletion of the genes of the major autolysin AcmA (55, 56) and/or AcmD (31) in *L. lactis* leads to a chaining phenotype (31). Disruption of *L. lactis galE,* encoding one of the enzymes participating in galactose utilization via the Leloir pathway, results in the formation of long chains of cells when they are grown on glucose as the sole carbon source, possibly due to a deficiency in cell separation (57). Formation of long chains also occurs by removal of *dltD*, a gene involved in the D-alanyl transesterification of LTAs in *L. lactis* (58). Mutation of *dltD* in *L. lactis* resulted in increased cell lysis due to strong reduction in D-Ala substitution in LTA.

Furthermore, an *dltD acmA* double mutant of *L. lactis* results in postponed cell lysis compared to normal cells, indicating that *dltD*-induced lysis depends on AcmA: a lower quantity of D-alanine on LTA decreases the degradation of AcmA by the cell wall protease HtrA, and consequently increases cell lysis (26).

Cell aggregation or cell clumping plays an important role in the attachment of bacteria to surfaces (59, 60) and in the formation of biofilms (61, 62). Cell aggregation or clumping can be governed by interactions between lectins and polysaccharides (50), by pili (63, 64), by external factors like chemicals (65), or by genes encoding cell surface-active molecules (60, 66–68). Some of the *L. lactis* genes that are known to alter cell aggregation are: *cluA* - located on the sex factor of *L. lactis* MG1363 (67, 69) and encoding a cell surface-bound protein ; *aggL* – a gene present on plasmid pKP1 specifying a "collagen-binding superfamily protein" responsible for aggregation of *L. lactis* subsp. *lactis* BGKP1, which was isolated from an artisanal semi-hard homemade cheese (66); *yhgE* and *yhhA* of the pilus gene cluster of *L. lactis* IL1403 (*yhgCDEyhhAB)* responsible for cell auto-aggregation and cell chaining when they are overexpressed (64).

Methods used to determine microbial cell surface properties

The cell surface and its decoration determine properties such as cell charge and hydrophobicity. A number of methods employed to determine the basic properties of microbial cell surfaces are listed in Table 2. The most widely used methods measure microbial adhesion to hydrocarbons (MATH) and the zeta potential or electrophoretic mobility of cells using a so-called ZetaSizer. MATH measures cell surface hydrophobicity, while the ZetaSizer allows estimating the surface charge (also called zeta potential) of cells. Using both methods it was shown that strain of *L. lactis* subsp. *lactis* had lower isoelectric points (pI, the pH at which the net surface charge is 0) than *Lactobacillus helveticus;* above pH 5 the lactococcal electrophoretic mobility was four times larger than that of *Lb. helveticus* (70). Similar results were obtained for cells from the exponential or stationary phase of growth, indicating that the cell surface of *L. lactis* is more hydrophilic than that of *Lb. helveticus*. The fact that the *L. lactis* surface is

more negatively charged than that of *Lb. helveticus* is probably related to the absence of an S-layer in the former, resulting in a higher exposure of cell wall components. The polysaccharide-to-protein ratio in the cell wall decoration increases from exponential to stationary phase in *Lb. helveticus* (70). This was hypothesized to be caused either by S-layer fragmentation or by PS or (L)TA protrusion through the S-layer. The gene *cbs*A encoding the collagen-binding S-layer protein of *Lactobacillus crispatus* JCM5610 (71) might be a candidate protein involved in the cell surface hydrophobicity in this species.

The net surface charge of bacteria is negative (36). Usually, the pI of hydrophobic bacterial strains is around pH 4 to 5, while relatively hydrophilic ones have lower isoelectric points (e. g. around pH 2) (72, 73). The pI of *Lactobacillus* species positively correlates with the concentration of nitrogen-containing groups on the cell surface while it was inversely correlated with the concentration of oxygen-containing groups (74). No correlation was found between surface charge and hydrophobicity (72, 75); however, it was hypothesized that for higher cell surface hydrophobicity glyco-proteinaceous surface components and LTA need to be formed, while polysaccharides are required for higher cell surface hydrophilicity (72, 76). Thus, polysaccharides, TA, and LTA seem to endow a bacterial surface with hydrophilic properties, whereas cell wall proteins and pili seem to provide hydrophobic attributes.

Table 2. Methods to determine microbial cell surface properties

Method	Description	Advantages	Disadvantages	Reference
Cell surface hydrophobicity (CSH)				
MATH Microbial Adhesion To Hydrocarbons	Cell suspension in buffer is mixed with hydrocarbon. Biomass migrating to hydrocarbon phase is calculated by measuring optical density decrease in aqueous phase	Easy, fast, low material costs	Low-throughput	(77–79)
MATH Kinetic version	Adhesion to hydrocarbon is followed as a function of time	Easy, low-cost	-	(80)
MATS Microbial Adhesion To Solvents	Similar to MATH but solvents are more polar (e.g. hexadecane, chloroform)	Easy, fast	Low-throughput, use of corrosive chemicals	(81)
HIC Hydrophobic-Interaction Chromatography	Measurement of concentration of trapped hydrophobic cells in octyl-sepharose column	Separates hydrophobic and hydrophilic bacteria	Not useful when cells tend to agglutinate	(82)
SAT Salt Aggregation Test (plus its kinetic version)	Turbidity is monitored upon aggregation of hydrophobic bacteria mixed with ammonium sulfate	Easy, low material costs, high-throughput	-	(65, 83)
CAM Contact Angle Measurements	Angle of water droplet on dried lawn of bacteria is proportional to their hydrophobic value	Predicts adhesion of bacteria to hydrophobic surfaces	Water droplet may rapidly hydrate hydrophobic bacterial lawn	(84, 85)
SDG Sucrose Density Gradient centrifugation and subsequent bacterial DNA quantification	Measures adhesion between cells and milk fat globules membrane (MFGM)	Allows measuring binding capacity of bacteria to different materials	Time consuming, more difficult than MATH	(86)

Cell surface charge				
MATS Microbial Adhesion To Solvents assay	Assesses electron-donor/ electron-acceptor (acid-base) properties of bacteria and predicts their adhesion behavior to solid surfaces in an aqueous system	Easy, fast	Low-throughput, uses corrosive chemicals like chloroform, diethyl ester, ethyl acetate	(81)
ME Micro-Electrophoresis	Measured electrophoretic mobility is re-calculated to zeta potential using Helmholtz-Smoluchowski equation.	Allows distinguishing cells with different charge within one (mixed) population	More sophisticated than ZP determination using ZetaSizer	(87)
EM or ZP Electrophoretic mobility or zeta potential measurement	Zeta potentials are calculated by measuring bacterial electrophoretic mobility in electric field (ZetaSizer)	Simple, easy, fast Detection of different bacteria in mixed sample possible	pH, ionic strength, concentration of bacteria in sample may all have effect on results	(88)

Microbes in industrially relevant processes

Microorganisms play major roles in industrial and pharmaceutical fermentation processes. In some of these applications such as the production of fermented foods, the degradation of woody materials (straw) or the bioremediation of soil bacterial attachment might be important. Below we discuss the potential role that bacterial surface properties may play in industrial fermentation processes.

Biofilm formation

Various microbes are able to form biofilms, microbial communities on a surface and surrounded by extracellular polymeric substances such as polysaccharides, glycoproteins, proteins, glycolipids, cellulose, and extracellular DNA (e-DNA). Based on the matrix composition, several functions have been ascribed to it: 1) the presence of polysaccharides allows to retain water, 2) e-DNA facilitates horizontal gene transfer, and 3) protection against unfavorable environmental changes (89, 90). At the start of formation of a biofilm, during initial cell attachment, Brownian motion, hydrogen bonding, and electrostatic forces all play a role (36, 91). In that stage, a surface-attached cell may still easily detach. In case the cell stays attached, hydrophobic forces as well as dipole-dipole, hydrogen-, ionic-, and covalent bonding become more important (36, 91) and the cell ultimately becomes attached "irreversibly". Bacterial contact with the surface takes place via fimbriae, pili, flagella and/or EPS (61). Once a cell is irreversibly attached it starts to divide and produce offspring, might attract other bacteria and, together, the community of cells start to produce different polymeric components and the biofilm matrix is formed. The biofilm matrix in turn accelerates further bacterial adhesion (47, 91).

Microbial adhesion and subsequent biofilm formation is an unfavorable process in many industrial and medical applications because bacteria can cause corrosion, serious illnesses, and lead to economic losses (47, 52, 90, 92, 93). In the food industry biofilm formation leads to increased processing costs or it can result in food poisoning (90, 91, 94). In the dairy industry, for example, biofilms can form in pipes, on conveyor belts, on floors, in plate-heat exchangers of pasteurizers, and on rubber seals (61). Specifically in plate-heat exchangers biofilms are formed on a fouling, which is deposits of salt, proteins and fat. Fouling reduces heat transfer through milk, and formed biofilm results in a poor quality, safety and shelf life of a product (95). Milk proteins such as whey proteins can either increase bacterial attachment to stainless steel, rubber, glass surfaces and leads to biofilm formation (96) or they conversely may prevent attachment of some microorganism (e.g. *Listeria monocytogenes* and *Salmonella typhimurium*) such as is the case for beta-lactoglobulin and casein (97). Biofilms in food industries like the

poultry, fish, meat processing industry as well as in dairying have been recently reviewed by (98).

Solid-state fermentation and degradation of plant material

Solid-state fermentations (SSF) are fermentations of solid surfaces/substrate with limited amounts of free water, but enough to support microbial growth. Fungi and yeasts are more frequently used than bacteria as fermenting organisms in such processes. Bacteria are mainly used in composting and plant food fermentations, in trickling filters for waste water treatment and also for waste gas treatment (99–101). Examples of SSF are bioremediation, biodegradation, biological detoxification of agro-industrial residues, biotransformation of plant residues for nutritional enrichment, production of antibiotics, enzymes, organic acids and amino acids, biofuel (102, 103), biosurfactants, biopesticides, and aroma compounds (104–108). The main microorganisms used in the SSF were reviewed in (104, 107) and with a focus on food fermentations (109, 110). A common example of SSF with LAB is silage fermentation (111, 112) where the produced lactic and acetic acids cause a pH reduction that prevents unwanted growth of *Clostridia* (113). The macromolecular matrix of the substrate in SSF is also important (104) as the degradation of e.g. cellulose or lignocellulose demands specialized enzyme activities that often need to be extracellular. Microbes like *Clostridium* species, *Acetivibrio cellulolyticus* and *Ruminococcus flavefaciens* have evolved special enzyme complexes on cell surfaces called cellulosomes, which allow the bacteria to adhere to and degrade plant cell walls (114, 115).

Bacterial adherence in non-food environments

Environmental, biomedical and industrial application of low molecular weight surface-active compounds, biosurfactants reducing a surface tension at air-water and oil-water interfaces, produced by bacteria were reviewed in (116); it was concluded that due to antimicrobial, anti-adhesive and immune-modulating properties microbial biosurfactants can be successfully applied in the biomedical industry. Biosurfactants are hardly applied in environmental applications like bioremediation of hydrocarbons because of the poor understanding of mechanisms of interactions between bacteria,

hydrocarbons and bio-surfactants (117). From the perspective of microbe-substrate interactions, three parameters are important during hydrocarbon polymer biodegradation: 1) microbial properties like surface hydrophobicity, expression and regulation of genes, adaptation to environmental conditions; 2) hydrocarbon properties like solubility in water and volatility, molecular complexity, toxicity, surface area, presence of other organic compounds; and 3) environmental parameters such as pH, water activity, temperature and nutrient availability (118). Bacterial adhesion to the hydrocarbon substrate does not always correlate with substrate degradation and bacterial growth (118). However, the addition of certain components can enhance hydrophobic interactions between bacteria and a substrate, such as ammonium sulfate (78), cationic surfactants like cetylpyridinium chloride (119), cationic polymers like poly-L-lysine or chitosan (118, 120), long chain alcohols like 1-decanol (121) or 1-dodecanol (122, 123). Bacteria are used for soil bioremediation (124–129) and cell size was reported to be the main determinant for bacterial transport through soil but no correlation was found between hydrophobicity and surface charge (130). Addition of surfactants to the soil material reduces bacterial adhesion to hydrophobic soil particles (131). However, surface hydrophobicity and positive charges on the cell surfaces contribute to the adhesion of bacteria to mineral particles, which are usually negatively charged (132).

Microbe-host interactions

There is increasing evidence that bacterial surface decoration plays an important role in the interactions of microbial cells with the human gut (133–135). The importance of the bacterial cell wall and its components in probiotic-immune interactions has been recently reviewed by Lee *et al.*(136). One of the examples is a *Lb. plantarum dlt* mutant that is not able to insert d-alanine into its TAs. The LTAs of the mutant were three times longer and displayed an increased degree of glucosylation (137), which impacted the immunomodulating capacity of the mutant strain relative to the wild type. The mutant cells displayed a strongly reduced induction of Toll-like receptor-2-dependent proinflammatory responses and a related increased stimulation of interleukin-10 (IL-10) in blood-derived immune cells (133). In a different example the expression of the

receptor-binding region SlpA of the S-layer protein of *Lb. brevis* ATCC8287 in *L. lactis* NZ9000 allowed the otherwise non-adhesive cells to adhere to human intestinal epithelial cells *in vitro* (138).

A description of molecular mechanisms involved in interactions between pneumococcal surface protein A (PspA) and human lactoferrin (hLf) was provided by Jedrzejas (2006). PspA is a negatively charged surface protein of *S. pneumonia.* PspA attaches non-covalently via its C-terminal choline-binding domain (CBD) to TAs of the cell wall or to LTAs of the cytoplasmic membrane (139). Due to its high negative charge PspA is repelled from the negatively charged capsule of *S. pneumonia*. hLf is a ligand for PspA antigen molecule. hLf exists in two forms: *apo*hLf, which does not contain ferric ions and *holo*hLf that contains Fe^{3+} ions. *apo*hLf possesses bactericidal anti-pneumococcal properties because it can bind Fe ions necessary for bacterial growth. Part of the PspA protein is positively charged and attaches to the negatively charged *apo*hLf; as a result *apo*hLf cannot bind Fe ions anymore and the bacteria escape the bactericidal properties of lactoferrin (139). Another example demonstrated that surface protein A (SlpA) of *Lb. acidophilus* NCFM is recognized by the fucose-recognizing lectin called AAL and the mannose-recognizing lectin concavalin A (ConA). This evidence supports a role for SlpA as a ligand for lectin dendritic cells-specific ICAM3 *(Intercellular adhesion molecule 3)*-grabbing nonintegrin receptor (140), which is involved in modulation of dendritic and T cell functions.

Observing the literature it can be concluded that more understanding of microbe-substrate interactions at molecular level occurs in pharmacological, biomedical research than in industrial fields.

Microbes in food matrices

LAB are used broadly to ferment milk, vegetables and other plant materials, and grains (141), which is done either in a liquid or a (semi)solid environment with significantly different matrix properties.

Milk and fermented milk matrices

Milk is a complex matrix consisting of proteins, carbohydrates, fat, minerals, and vitamins. Milk proteins consist of the so-called whey proteins (β-lactoglobulin, α-lactalbumin, bovine serum albumin (BSA), immunoglobulin (Ig), and proteose-pepton) and caseins (αs1-, αs2-, β-, κ-caseins) and miscellaneous proteins (e.g. proteins in the milk fat globule membrane) (142–144). Caseins are present in casein micelles with an average size of 200 nm. Whey proteins are very structured, while caseins have a more open structure and are highly flexible (145). All caseins contain phosphorus, and thus, due to their phosphorus content, caseins can withstand heating to up to 100°C for 24h (146). Serum proteins do not contain phosphorus and are completely denatured when kept at 90°C for 10 min. Upon heat treatment whey proteins attach to the casein micelles (147, 148). Caseins possess hydrophobic properties, which decrease in the order β- > κ- > α_{s1}- > α_{s2}- casein (143). β-casein is a strong emulsifier (149). The κ-casein molecule is located on the surface of the casein micelle with its hydrophobic C-terminal region sub-merged in the casein micelle, and it glycosylated N-terminal region protruding from the micelle. Thus, κ-casein provides hydrophilic properties to casein micelles (143). Fat in milk is present predominantly in spherical droplets ranging from 0.2 to 15 μm in diameter which are surrounded by a milk fat globular membrane (MFGM) (150, 151). Attachment of microbial cells, due to their surface hydrophobicity, to MFGM has been reported (86).

Lactose is a disaccharide used by milk starter culture bacteria as an energy source for growth. Its degradation leads to the production of lactate and a consequent pH decrease. When the pH reaches 4.6, the pI of caseins, this results in casein coagulation and the formation of a gel-like matrix. Next to acidification, LAB can play an important

role in flavour and taste formation. During cheese manufacturing the added proteolytic enzyme rennet (chymosin) removes the negative charges of casein micelles by cutting the hydrophilic part of κ-casein, thus causing the formed para-casein protein to coagulate into a casein gel (curd) through Van der Waals-, steric-, and hydrophobic forces (152). Thus, the *cheese* matrix consists of aggregated proteins with embedded fat droplets, air droplets, starter cells, and whey pockets. Rennet is not added during yoghurt production and in a *yogurt* matrix in addition to aggregated proteins with embedded starter culture cells, fat droplets, serum regions, the exopolysaccharides are the additional structural component.

Vegetable matrices and plant materials

Silage is a fermented product of grass or other plant material obtained with anaerobic microorganisms. LAB generate lactic and acetic acids from sugars of the raw material (113, 153). The substrates in such plant matrices are not or only poorly soluble and, therefore, extracellular enzymes are required for their release (e.g. the enzymes form a so-called extracellular cellulosome (114, 115)). Surface-attached bacteria are the inoculum in wild fermentations (154). More information on silage fermentation in SSF was presented above. Fermented foods like sauerkraut, olives, or pickles use LAB species similar to those employed in silage production.

Bread. The bread dough matrix consists of a gluten network with starch and incorporated air droplets (155). LAB are used to reduce the pH of the dough, to improve the shelf life of bread (156), to create flavour and, partially, taste (157) and to produce EPS in order to replace the hydrocolloids used as texturizing and antistaling agents (158, 159).

Meat. LAB are used in the fermentation of certain meats in order to obtain a safe, tasty and high-quality product (160–162). The myosin and actin proteins are responsible for the formation of the matrix of fermented sausage (163). Indeed, after meat chopping and salt addition, the meat cells' integrity is destroyed and myofibrils absorb salt, which leads to an acceleration of protein disintegration. Due to the hygroscopic properties of salt, water penetrates between the protein molecules and causes myofibril swelling and

further disintegration of bonds between actin and myosin. During the fermentation of the available sugars by LAB and the consequent formation of lactic acid, water-soluble myofibrillar proteins and connective tissue proteins form a further 3D network with entrapped fat and meat particles. After sausage acidification and during drying, the bonding between proteins and, thus, the protein network becomes stronger, which eventually results in structural firmness of the sausage. It was shown that *Lactobacilli* grow in aggregates within the sausage matrix (163). Other microstructures were described for certain Polish sausages and for reduced-sodium frankfurter sausages (164), but overall there is very limited information on the localization of LAB in the fermented meat matrix.

Forces involved in interactions between microbes and the food matrix

For adhesion of bacterial cells to a food component the properties of both surfaces, that of the component and that of the cells, are important. For example, the more porous, rough and hydrophobic a surface is, the better and faster bacteria adhere to it (49, 50, 165). Most bacterial cells are negatively charged (36) but their surface charge varies with growth conditions (165). Interactions between bacteria and food surfaces/particles will depend on the molecules present on the bacterial cell wall; they can be either chemical (covalent bonding, van der Waals-, electrostatic-, or hydrogen bonding), of dipole nature (dipole-dipole, dipole-induced dipole, ion-dipole), or hydrophobic (36), (166). Electrostatic interactions will depend on the pH and the ion concentration in the surrounding environment. Hydrophobic forces arise between apolar matrix molecules and bacterial surface structures such as LPS, fimbriae, pili, or cell wall-located proteins. Hydrophobic forces are much stronger than hydrogen bonds, electrostatic forces or covalent bonds (167) and, therefore, they may play a major role in adhesion of bacteria to surfaces. Steric interactions can occur via overlapping of regions in polymeric molecules that are on bacteria and on matrix surfaces. If the concentration of polymers is high, repulsion between the bacterial and matrix surfaces can occur as a consequence of polymer saturation on both surfaces. If the polymer concentration is low, steric attraction can occur between a bacterium and a matrix

surface and polymers play the role of connector between the two (36). Steric polymer interactions are the cause of microbial aggregation (168).

Although many bacterial cell surface decoration molecules are known and some of the genes involved have been described, there is still only limited knowledge as to how the surface properties of a bacterial cell are eventually determined. It is not clear how and why cell surface properties change as a function of the growth phase. Genomics can help to understand the interactions between bacterial cell surfaces and abiotic or food matrix surfaces. Genome sequences alone are not enough to predict bacterial responses to certain environmental conditions but transcriptome-phenotype correlation analyses and next-generation or whole-genome sequencing methods, and sRNA sequencing may offer powerful additional approaches in understanding microbe-matrix interactions at the level of gene regulation.

Interactions between LAB and major milk components

A key functionality of the dairy starter culture is fermentation: to preserve food through a decrease in pH. Some LAB species produce bacteriocins like nisin or plantaricin, which prevent outgrowth of *Listeria* (169). During cheese ripening LAB degrade proteins to peptides and amino acids, some of which are (volatile) flavours or flavour precursors while others might cause an off-flavour if for example too many bitter peptides are formed. In Gouda and Cheddar-type cheese matrices a weak lipolysis, the degradation of fat to fatty acids by enzymes produced by starter culture bacteria, is desirable albeit that a too strong lipolysis can cause a rancid/soapy off-flavour (170–172). In a hard cheese matrix kept for a long time (e.g. over 4 months) and if bacterial cells appear close to the fat droplets, the bacterial enzymes can sometimes extensively degrade fat membranes leading fat globules to partly fused. The resulting continuous fat phase is undesirable (173). On the contrary, for the production of Casín cheese, a Northern Spain traditional cheese, the curd is manual kneaded daily for a week and then weekly up to the end of ripening in order to break the fat droplets and to achieve full texture uniformity (174). The resulting strong lipolysis provides a spicy, pungent, bitter, strong flavour taste that is much desired in this type of cheese (174). The use of

specific microorganisms can also determine the texture or mouthfeel of a product. Microbial polysaccharides such as EPS can increase the viscosity and firmness of fermentation products or cause a decrease of syneresis, the unwanted appearance over time of free water. For instance, yogurt is made with *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, two EPS-producing LAB species (175). EPS is important in yogurt consistency, ropiness and viscosity after stirring (176). When the yogurt structure was examined by confocal laser scanning microscopy, the EPS were observed in the pores in the gel network, separated from the coagulated proteins; this is probably due to incompatibility between EPS and protein aggregates (177). The proteolytic *Lb. bulgaricus* enhances the growth of the auxotrophic *S. thermophilus* by degrading casein. On the other hand, *S. thermophilus* produces formic acid and folate, which support the growth of *Lb. bulgaricus*. Literature suggests that the two species adhere to each other in yoghurt, but evidence is lacking (178). By varying the ratio of the species, or the specific strains used, folate and vitamin B12 might be enriched in yogurt, or it may result in specific aroma formation. During fermentations also nutraceuticals, components that contribute to the human health via a specific physiological action, can be formed (141).

As may be appreciated from the above, the location in the fermentation broth as well as the site at which they are ultimately included in the final product might play an important role in the functionality of the LAB and the success of the fermentation process. The location in dairy foods of LAB has up to now been mostly studied in cheese and with the major starter culture bacteria used in cheese making, *L. lactis*. The position of the bacteria in the cheese matrix is determined during the coagulation step in cheese production. Bacteria appeared in the cheese matrix next to protein molecules, or close to fat droplets. Such a distribution of the bacterial cells could be related to their surface properties. Cells of *L. lactis* were observed around fat droplets, or in whey pockets (179, 180).

Interaction between LAB and milk proteins

Homogenized milk, butter, cream cheese and mayonnaise are food emulsions. LAB are used in some of these products. Surface characteristics of *L. lactis* were examined in relation to their interaction with milk proteins (sodium caseinate, whey protein concentrate (WPC) and whey protein isolate (WPI)) and stability of emulsions (73, 181, 182). In general, casein proteins are negatively charged at pH 7 and positively charged below their pI of 4.6 (183). Most LAB cells are negatively charged at a pH in the range of 3 - 7. Therefore, emulsions made with sunflower oil and stabilized with different types of milk proteins (WPC, WPI, sodium caseinate) are instable when a bacterial suspension was added because the proteins covering the fat droplets had a charge opposite to that of the bacterial cells (182). At pH 3 oil droplets covered with WPC or WPI proteins aggregated after addition of *L. lactis* LLD16 due to electrostatic attraction between the negatively charged bacteria and positively charged protein-coated oil droplets and thus caused emulsion instability (182). Strain specificity also plays a role in emulsion stability. In contrast to strain LLD16 which is constantly negatively charged independently of pH, the strain LLD18 is positively charged below its pI of 3.5 and negatively charged at pH above 3.5. Therefore, stable emulsions were observed at pH 3 when oil droplets, stabilized by positively charged proteins of WPC, were mixed with the positively charged *L. lactis* subsp. *lactis* strain LLD18 (182). At pH 7 negatively charged bacteria (LLD18) can also interact with negatively charged oil droplets via bridges of Ca^{2+} ions. In this case the order of component addition is important: if bacteria and cations were mixed before the emulsion was added, the final emulsion was stable (182). In the same study it was shown that hydrophobic interactions between bacteria and proteins can also lead to attachment of bacteria to oil droplets but these interactions do not lead to emulsion stabilization (182). $CaCl_2$ concentration and pH have an effect on the stability of soy bean oil-in-water emulsions (184). At low pH, $CaCl_2$ decreases the positive charge of fat droplets covered by WPI proteins, while at high pH it decreased the negative charge of these droplets. Also, the zeta potential of fat droplets decreases upon addition of $CaCl_2$ (184). When relating this knowledge to the cheese matrix, it may be concluded that pH and the concentration of certain ions

(sodium, phosphorus, calcium) might influence bacterial interactions with proteins. Indeed, in cheese renneted at pH 6.6 the matrix was porous with a dense protein network with fat droplets of different sizes distributed in the aqueous phase, whereas rennet addition at pH 5.2 led to a more homogeneous cheese matrix with a less dense protein network and evenly distributed fat droplets (185). Bacteria in cheese renneted at pH 6.6 were organized in colonies after 1 day of incubation, while in cheese renneted at pH of 5.2 they were evenly distributed as single cells in the protein network in the cheese. Additionally, lysis of bacterial cells in cheese renneted at low pH occurred later than in cheese renneted at pH 6.6, thus, the cell lysis induction was suggested to be caused by alteration in cheese microstructure and in cell localization (185). It seems that cell lysis could be caused also by the pH itself, or by a combination of factors mentioned above.

The cell surfaces of eight strains of three different LAB species, namely *Lactobacillus casei* ssp. *casei*, *Lb. paracasei* ssp. *paracasei*, and *Lb. rhamnosus*, appeared to be relatively hydrophilic but despite this, the bacterial cells could still adhere to apolar components like fat droplets (72). Furthermore, lactobacilli seem to have a strong basic and weak acidic character as they show affinity for acidic solvents like chloroform and have a low affinity for the basic ethyl acetate. Their basic character can be explained by the presence of carboxylic groups on the surface of PG and the S-layer. That is why these bacteria tend to donate electrons. Additionally, their hydrophilic surface might be caused by cell wall polysaccharides, which could provide OH^- groups interacting with H^+ ions of water. The interactions of *Lb. rhamnosus* strains GG and GR-1 with dairy proteins (casein micelles, native and denatured whey proteins) were studied by measuring adhesion forces using atomic force microscopy at different pHs (179). *Lb. rhamnosus* GG showed a stronger specific affinity to denatured whey proteins than *Lb. rhamnosus* GR-1, whereas both strains non-specifically adhered to casein micelles. Apparently, matrix-microbe interactions are strain-specific (179). These observations were also related to pH and the structure of the proteins employed: casein micelles are porous and their inner part is only accessible to small molecules, whereas whey proteins have a defined tertiary structure that is accessible to biomolecules (179).

EPS produced by LAB is known to modify food texture and increase water retention in fermented dairy products. Very little is known, however, about the mechanisms governing the textural changes observed during product manufacturing. Usually EPS are loose from the microbial cells, and in the milk matrix the association between casein micelles and bacterial EPS is electrostatic in nature (186). EPS structural parameters like the type of charged groups, charge density, molecular weight and the molecule flexibility can influence the forces of adhesion to milk proteins, and can influence on solubility of complexes with whey proteins than with casein micelles (187). Some EPS can be connected to PG without covalent bonding (14). Bacteria in which this is the case can interact with milk proteins (caseins and whey proteins) via filamentous strands (EPS) as was observable in buttermilk and milk permeate inoculated with the EPS producing *L. lactis* ssp. *cremoris* JFR1 (188). Buttermilk also contains all major proteins as well as fragments of the milk fat globule membrane, while milk permeate consists of lactose, vitamins and minerals. In the reported study, whey proteins were added to milk permeate. Scanning electron microscopy revealed that EPS, emerging from the milk-grown bacteria in the form of strings, attached the bacteria to aggregated milk proteins. The amount of whey protein in whey permeate positively influenced the amount of EPS formed by the bacteria. In milk permeate supplemented with whey proteins, EPS was also attached as long strings to bacterial cells and intertwined with whey protein aggregates, thus connecting the bacterial cells to the proteins.

Certain species like *Lactobacillus, Streptococcus*, *Bifidobacterim* and in smaller level *Lactococcus* strains of LAB are used as probiotics, living organisms believed to provide health benefits when consumed as food supplement (189). Food-grade polymeric matrices e.g. of calcium alginate and k-carrageenan are used for immobilization of probiotics. However, full mechanical stability of capsules and controlled release have not yet been achieved (190, 191). As an alternative solution, the complexes that LAB can form with whey proteins and k-carrageenans can be used as structural elements and as probiotic carriers in functional foods such as yogurt, fermented lactic beverages, or cheese (192). *Lb. plantarum* was entrapped in a complex of WPI and k-carrageenan.

This complex protected the bacteria from low pH and increased cell viability. Hydrophobic as well as electrostatic forces were responsible for the formation of bacteria-whey protein complexes. The whey proteins with attached *Lb. plantarum* cells were then used to form complexes with k-carrageenan via electrostatic interactions (193), resulting in a bacteria-WPI-k-carrageenan complex coacervates (192). No clear underlying molecular mechanism for the bacteria-WPI-k-carrageenan interactions was provided. Immobilization as well as cryoprotection is of importance during freeze drying of probiotic bacteria to protect viability. Sucrose (10%) (194), sorbitol, trehalose and reconstituted skim milk (195) and poly-γ-glutamic acid (196) are all being used as cryoprotectors of probiotic strains but the exact mechanism of cryoprotection is not known.

Interaction between microbes and fat droplets

Several components of the milk fat globule membrane (MFGM) are also present on the cell surfaces of LAB e.g., carbohydrate chains, proteins, glycoproteins, enzymes, and phospholipids. Hence, we hypothesize that electrostatic interactions in milk between bacterial surfaces and the MFGM of fat droplets could in principle take place via interactions of S-layer proteins (in *Lactobacillus* strains) with carbohydrates of the MFGM or of bacterial EPS with MFGM proteins. Hydrophobic interactions between cell surface components and MFGM molecules can overtake electrostatic ones e.g. when the environmental pH is close to the pI of the bacteria, or when the suspension contains high ionic strength to suppress electrostatic interactions. In addition, loss of oligosaccharide components from the cell surface results in an increase in the hydrophobicity of cells, as was demonstrated for *E. coli* rough mutants that displayed an enhanced affinity to hydrocarbons (77).

Attachment of LAB to lipids can be interesting for both the formation and retention of flavour compounds in the food matrix (73, 197, 198). Such a function of LAB might be important in low-fat cheeses where the ratio between hydrophilic and hydrophobic components is different from that in high-fat cheese matrices. In general, aroma compounds have a poor affinity for hydrophilic components and, thus, flavour

compounds may escape from low-fat cheeses. Using LAB with increased hydrophobic surface properties may help to retain flavour components in the food matrix by binding them. Only few studies have dealt the possible interactions between aroma compounds and microbial cell surfaces (197, 199). A study on yeast showed that the hydrophobicity of wine volatiles (isoamyl acetate, hexanol, ethyl hexanoate, b-ionone) and the conformation and composition of mannoproteins (presence of glycosyl residues, glycosyl-linkage composition, protein content) play important roles in yeast cell wall aroma retention (199, 200). To understand the molecular details of the interactions proved difficult as glycosidic as well as peptidic parts of the mannoproteins may interact with aroma components (199). The surface of LAB was shown in suspensions and emulsions to interact directly or indirectly with aroma compounds, promoting the distribution of volatile compounds (197). For example, when hydrophobic aroma compounds of cheese (ethyl acetate, ethyl hexanoate) were added to an emulsion before the LAB were included, the aroma molecules can enter the oil droplets. Subsequently, the LAB cells cover the oil droplets and form a barrier that prevents the release of aroma from the lipids (197).

Fat droplets can be covered with ionic surfactants, compounds that lower the interfacial tension between two liquids, such as hexadecyl-trimethylammonium bromide (CTAB), sodium dodecyl sulfate (SDS) and Tween 20, in order to stabilize oil-in-water emulsions (73). When LAB were mixed with fat droplets covered with one of the surfactants, the emulsion became instable when the charge of the bacteria was opposite to that of the droplets (73). As a result, the fat droplets flocculated. Such emulsion destabilization was reduced by adding 100-200 mM NaCl. Possibly, the negative surface charge of the bacteria is neutralized by Na^+ ions and as a result, electrostatic repulsion played a major role between the positively charged fat droplets (covered by surfactant) and the bacterial surfaces. The effect of bacteria on emulsion stability is strain dependent (73).

It was recently shown that bacterial physicochemical surface properties play a significant role in the spatial colonization of food matrices by the bacteria as well as their metabolic properties in the food (180). Spatial colonization should be studied for each

strain or species in a starter culture in order to determine if bacterial cells could meet certain probiotic, organoleptic or technological requirements. For example, from the four *L. lactis* strains LLD16, LLD18, LB340, TA60, strain LLD16 appeared in the serum phase if the milk pH was 6.5 while it was present in the lipid phase at a pH of 3. *L. lactis* LLD18 always appeared at the interphase between the lipid and serum phases (180). Colonization of the milk matrix during fermentation was also investigated: in the beginning of the fermentation process the cells of all strains were evenly dispersed in the milk, but later high strain-specific cell densities were observed in specific places in the acidified and clotted milk matrix. Strain LLD18 was mainly present in the protein gel while strains LLD16, TA60 and LB340 appeared in the lipid layer (180). The authors suggest that spatial distribution of specific LAB strains and the subsequent further colonization of the matrix might have a great impact on development in the product of taste, color, flavour, etc.

Fat droplets and their quantity on the other hand influence the retention of bacteria in the food matrix. Higher LAB retention levels were seen in high-fat cheese curd than in low-fat curd (201). Bacteria were mostly present in close proximity to and around the fat droplets and clear interactions were detected, as the MFGM appeared to change with time at the contact area with starter cells. The deformation was proposed to be caused by proteolytic activity of bacteria degrading caseins in the proximity of the fat droplet, which would weaken the casein matrix, impeding it to counteract the pressure of the fat droplets upon which the MFGM is proposed to stretch. The authors also hypothesized that, since cells lyse during cheese ripening (202), the remaining cell wall particles or cell ghosts could directly contact the inside of fat globules or be integrated into the MFGM (201).

All in all, microbial surface structures are highly diverse and surface properties are of importance for the physical adhesion to (semi)solid substrates in industrial food fermentations. Such microbe-matrix interactions could be crucial for the success of these fermentations. Cell morphology and surface properties such as charge, hydrophobicity, the presence of EPS, pili, and proteins influence the interactions between bacteria and the food matrix. Attachment of bacteria to matrix components

can lead to changes in matrix composition and properties, partly also because of exposure to bacterial enzymes. On the other hand, food matrix surfaces also influence microbe-matrix interactions. In the food industry, altering the composition of the growth medium, temperature, pH, or ionic strength during fermentation can affect the physico-chemical and adhesive properties of cell surfaces.

This thesis investigates mechanisms that govern microbe-matrix interactions under conditions of milk fermentation using genome- and transcriptome sequencing, gene cloning technologies, genotype-phenotype matching as well as direct testing of *L. lactis* cultures in dairy applications. A better knowledge of the underlying mechanisms would allow a new way of steering starter culture functionality. This would broaden application possibilities to improve and diversify product texture, taste, and flavour and might ultimately result in the development of new products.

Outline of the thesis

In this thesis it was examined whether and how surface properties of the industrially important lactic acid bacterium *Lactococcus lactis* could influence industrial food fermentation (namely dairy) processes and reveal whether there might be hitherto unused potential to improve milk fermentation processes.

Chapter 2 presents the genome sequence of the dairy isolate *L. lactis* NCDO712. The results reveal that the strain carries, in addition to its circular chromosome, 6 rather than the 5 plasmids reported earlier. A new 50-kb plasmid designated pNZ712 encodes functional nisin immunity and copper resistance. The 16-kb plasmid pSH74 contains a novel and functional 8-kb pilus gene cluster.

Chapter 3 describes the surface properties (charge, hydrophobicity, emulsion stability, attachment to proteins) of 55 *L. lactis* strains from dairy as well as plant origin. The results show a high degree of biodiversity of these properties. Gene-trait matching and subsequent gene overexpression and deletion analyses allowed identifying three proteins involved in altered surface hydrophobicity and attachment to milk proteins. The data also show that *L. lactis* strains isolated from a dairy environment bind higher amounts of milk proteins than do plant isolates.

Chapter 4 demonstrates that cell surface alterations in *L. lactis* leading to cell chaining and clumping, as well as EPS production and pili expression affect the viscosity and/or gel hardness of milk fermentation products and the localization of cells in fermented milk. These observations are indicative of a cell surface-dependent potential of *L. lactis* cells as structure elements in fermented dairy products.

Chapter 5 shows that overexpression of pili on the cell surface of *L. lactis* cells can increase retention of the lactococcal cells in the curd up to 99% instead of the reported < 80%. The results also show that *L. lactis* cell surface alterations can strongly affect the distribution of cells in the cheese matrix. These data suggest that surface properties of bacterial strains making up a dairy starter culture can strongly determine their retention

and distribution in the cheese curd and thereby possibly affect cheese texture and ripening, as well as whey quality.

Chapter 6 demonstrates that lactococcal cells reside at the oil-water interphase upon mixing them with hydrocarbons thereby facilitating bacterial emulsification. The results showed that cell aggregation is of importance for the formation of oil-in-water emulsions. RNA sequencing of *L. lactis* cells that are present on the oil-water interphase revealed a response mainly of genes involved in amino acid and inorganic ion transport and metabolism.

Chapter 7 summarizes the most important findings of this thesis and future research perspectives are discussed.

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Chapter 2

Plasmid complement of *Lactococcus lactis* NCDO712 reveals a novel pilus gene cluster

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Abstract

Lactococcus lactis MG1363 is an important gram-positive model organism. It is a plasmid-free and phage-cured derivative of strain NCDO712. Plasmid-cured strains facilitate studies on molecular biological aspects, but many properties which make *L. lactis* an important organism in the dairy industry are plasmid encoded. We sequenced the total DNA of strain NCDO712 and, contrary to earlier reports, revealed that the strain carries 6 rather than 5 plasmids. A new 50-kb plasmid, designated pNZ712, encodes functional nisin immunity (*nisCIP)* and copper resistance (*lcoRSABC)*. The copper resistance could be used as a marker for the conjugation of pNZ712 to *L. lactis* MG1614. A genome comparison with the plasmid cured daughter strain MG1363 showed that the number of single nucleotide polymorphisms that accumulated in the laboratory since the strains diverted more than 30 years ago is limited to 11 of which only 5 lead to amino acid changes. The 16-kb plasmid pSH74 was found to contain a novel 8-kb pilus gene cluster *spaCB-spaA-srtC1-srtC2*, which is predicted to encode a pilin tip protein SpaC, a pilus basal subunit SpaB, and a pilus backbone protein SpaA. The sortases SrtC1/SrtC2 are most likely involved in pilus polymerization while the chromosomally encoded SrtA could act to anchor the pilus to peptidoglycan in the cell wall. Overexpression of the pilus gene cluster from a multi-copy plasmid in *L. lactis* MG1363 resulted in cell chaining, aggregation, rapid sedimentation and increased conjugation efficiency of the cells. Electron microscopy showed that the overexpression of the pilus gene cluster leads to appendices on the cell surfaces. A deletion of the gene encoding the putative basal protein *spaB* led to more pilus-like structures on the cell surface, but cell aggregation and cell chaining were no longer observed. This is consistent with the prediction that *spaCB* is involved in the anchoring of the pili to the cell.

Introduction

Lactococcus lactis is a gram-positive, non-pathogenic, non-spore forming lactic acid bacterium (LAB) that is often isolated from plant material or a dairy environment (1, 2). It is widely used in the dairy industry as a starter culture for the production of cheese, buttermilk and quark. Strains of *L. lactis* typically contain one to eight different plasmids (3, 4) ranging from 1 kb to more than 100 kb in size (5, 6). Examples are *L. lactis* SK11, a phage-resistant dairy strain used in cheese making that carries 5 plasmids (7), and *L. lactis* IL594, the 7-plasmid-containing parent of the plasmid-free laboratory strain IL1403 (8). The plasmids often specify traits of industrial importance such as the ability to grow on lactose, to utilize milk protein or stress resistance (9, 10). Other important plasmid-encoded functions include bacteriocin production (11, 12) and resistance (13, 14), metal ion resistance (15), antibiotic resistance (16, 17) and bacteriophage resistance (18, 19). A recent publication described a CRISPR-Cas system that was encoded by a lactococcal plasmid, and it was concluded to be functional (20). In addition, several genes related to lactococcal surface properties are carried on plasmids (21–23), such as *aggL*, a gene responsible for cell auto-aggregation, or genes responsible for adhesion to mucus (24).

One of the most intensively studied *L. lactis* strains is MG1363, a plasmid-cured derivative of strain NCDO712 (25, 26). NCDO712 was originally isolated from a dairy starter culture and was described to harbor 5 plasmids with molecular sizes of approximately 33, 9, 5.2, 2.5, and 1.8 MDa (26). During plasmid curing of strain NCDO712, derivatives carrying individual plasmids were obtained, allowing a targeted analysis of plasmid-encoded functions (26). Important biotechnological properties of the strain, namely lactose utilization and proteolysis, were linked to the 33 MDa (55 kb) plasmid pLP712 (26, 27).

This lactose/protease plasmid pLP712 (27) can be transferred to other lactococcal strains by conjugation (28). Conjugation can occur through co-integrate formation between pLP712 and a genome-encoded sex factor (SF). The co-integrate is roughly double the size of pLP712 since the SF is 59.5 kb (29). After conjugation

approximately half of the transconjugants displayed an aggregating phenotype and transferred the lactose/protease plasmid with high-frequency (28, 30–33). The aggregating phenotype is only seen in transconjugants carrying the pLP712-SF co-integrate (29, 34) and was linked to the *cluA* gene on the SF (34, 35). This gene encodes a surface protein involved in cell-to-cell contact and cell aggregation (34, 35).

Some *L. lactis* strains also express proteinaceous surface appendages called pili (21, 22, 24). Pili are known to have different functions in bacteria, including adhesion to surfaces (type I pili) or motility (type IV pili) (36–39). Pilus biosynthesis genes can be encoded by the chromosomal DNA (22) or by plasmids (21) and they are described to be involved in cell aggregation (40), bacterial adherence to host cells (41–43) and attachment to environmental substrates/surfaces (44).

Two plasmids of *L. lactis* NCDO712 have been sequenced. Plasmid pLP712 (55395 bp) harbors the genes for lactose import and catabolism, the extracellular protease PrtP, and genes encoding extracellular proteins, transposases, and hypothetical proteins (27). Plasmid pSH71, the smallest one (2062 bp) (45), is highly similar to pWV01 (45, 46) and both of them are the basis of an array of broad host-range cloning and gene expression vectors (45, 47–51).

Here we sequenced all plasmids of *L. lactis* NCDO712 and found that contrary to earlier reports, it contains not 5, but 6 plasmids (26). The additional plasmid encodes functional nisin immunity and copper resistance genes. Additionally, we identified a novel pilus gene cluster on plasmid pSH74, which we showed to be functional by overexpression and phenotypic analyses.

Materials and Methods

Bacterial strains, growth conditions and medium

L. lactis subsp. *cremoris* NCDO712 (26) and its derivatives (Table 1) were grown at 30°C in M17 (Oxoid, Thermo Scientific, Hampshire, UK). The lactose-positive *L. lactis* strains NDO712 and its derivatives SH4109 and MG1299 were grown in M17 containing 1% lactose (LM17), all other strains were grown in M17 supplemented with 1% glucose (GM17). When required, erythromycin (Ery; 10 μg/mL), chloramphenicol (Cm; 5 μg/mL), rifampicin (Rif; 50 μg/mL), or streptomycin (Str; 100 μg/mL) was added.

Table 1. Strains and plasmids used in this study.

Strain or plasmid	Characteristics[#]	Reference
	***L. lactis* strains**	
NCDO712	*L. lactis* dairy isolate (harboring pLP712, pSH71, pSH72, pSH73, pSH74, pNZ712)	(26) This study
MG1363	Plasmid-cured derivative of *L. lactis* NCDO712	(26)
SH4109	Prophage-cured derivative of *L. lactis* NCDO712 containing all 6 plasmids found in this study	(26)
MG1388	A phage T712 lysogen derived from *L. lactis* MG1363	(26)
MG1362	Derivative of *L. lactis* NCDO712 (harbors pSH72)	(26)
MG1063	Derivative of *L. lactis* NCDO712 (harbors pSH73 and pSH72)	(26)
MG1261	Derivative of *L. lactis* NCDO712 (harbors pSH73)	(26)
MG1365	Derivative of *L. lactis* NCDO712 (harbors pSH71)	(26)
MG1299	Derivative of *L. lactis* NCDO712 (harbors pLP712)	(26)
NZ9700	Nis^R; Derivative of *L. lactis* MG1363; *pepN::nisRK*	(48)
MG1614	Str^R and Rif^R derivative of *L. lactis* MG1363	(26)
IL1403	Plasmid-free derivative of *L. lactis* IL594	(1)
	Plasmids	
pIL253	Ery^R; 4.9 kb; Low copy-number derivative of pAMβ1	(52)
pIL253*pil*	Ery^R; 13.1 kb; pIL253 harboring pSH74 pilus gene cluster *spaCB-spaA-srtC1-srtC2* with 300 bp upstream region	This study
pIL253*pilΔ1*	Ery^R; 11.6 kb; pIL253 harboring *spaCB-spaA-srtC1-srtC2* with 1,5-kb internal deletion in *spaCB*	This study

[#] Str^R – streptomycin resistant, Rif^R – rifampicin resistant, Ery^R – erythromycin resistant, Nis^R – nisin resistant

DNA sequencing and sequence assembly

Total DNA of *L. lactis* NCDO712 was isolated using phenol-chloroform extraction as previously described (53) with the following modifications. Exponentially growing cells were harvested by centrifugation (10 min at 6240 g) after which the cell pellet was re-suspended in THMS buffer (30 mM Tris-HCL (pH 8), 3 mM magnesium chloride, 25% sucrose) containing lysozyme (2 mg/mL) and 50 µg/mL RNase and incubated for 1 h at 37°C. Subsequently, the cells were treated with SDS (at 1% final concentration) for 20 min at 65°C. Next, proteinase K (0.3 mg/mL) was added and incubation was continued for 10 min at 37°C. Total DNA was extracted from the lysate using several extractions with phenol/chloroform after which the DNA was precipitated with isopropanol, was dissolved in sterile water and stored at 4°C.

The purified total DNA was sheared to fragments of approximately 500 bp using the Covaris ultrasone device (KBioscience, LGC, Köln, Germany). The paired-end NEB NExtGen library preparation kit (New England Biolabs, Inc., MA, US) was used according to the manufacturer's instructions to prepare NGS libraries. The libraries were sequenced on an Illumina HiSeq2000 (Illumina, Inc., San Diego, CA, USA), providing paired-end sequences of 101 bases. Velvet (54, 55) was used in combination with VelvetOptimiser (http://bioinformatics.net.au/software.velvetoptimiser.shtml) to perform *de novo* paired-end assembly of the genome. All contigs that did not map to the published nucleotide sequence of the *L. lactis* MG1363 genome (Accession: NC_009004.1) were assumed to be plasmid fragments; these were first scaffolded by mapping onto known *L. lactis* plasmids in the NCBI database. Further scaffolding was supported by PacBio sequencing (BaseClear, Leiden, The Netherlands) on a 5-kb library of NCDO712 total DNA. Remaining gaps in the plasmid sequences were closed with dedicated PCR reactions followed by amplicon sequencing (BaseClear, Leiden, The Netherlands).

Initial automatic annotation of the plasmids was performed using the RAST annotation server (56). Manual curation of plasmid-encoded features was performed with Artemis (57, 58), followed by family, domain, motif and context analyses of encoded proteins

using BlastP (NCBI) and Interpro (http://www.ebi.ac.uk/interpro/). IS elements and transposase genes were identified using IS Finder (https://www-is.biotoul.fr//). The DNA sequences of the assembled plasmids were used for a BlastN search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) in the NCBI plasmid database containing complete plasmids. Determination of single nucleotide polymorphisms (SNPs) in the nucleotide sequence of the chromosome of strain NCDO712 relative to that of its derivative strain MG1363 was performed using the Breseq software package (59) and the GenBank file: NC_009004 in combination with corresponding next-generation sequencing data: SRA064225 as templates.

For non-synonymous SNPs the software SIFT (60) and the UniProt-TrEMBL database (http://www.uniprot.org/) were used to predict whether an amino acid substitution would affect protein function.

The nucleotide sequences of the pSH74 and pNZ712 plasmids were deposited in the NCBI GenBank database with accession numbers KX138410 and KX138409, respectively.

Determination of nisin and copper resistance

Overnight cultures of *L. lactis* were diluted in fresh GM17 medium to a final optical density at 600 nm (OD_{600}) of 0.03. To measure nisin resistance, nisin from *L. lactis* (N6764-5G, Sigma-Aldrich, Steinheim, Germany) was added to the medium to different end concentrations (0 - 20 ng/mL). *L. lactis* NZ9700 (Table 1) was used as a control. The strains were grown in 10 mL sterile tubes for 7 h at 30°C. The OD_{600} was measured after 4 h and after 7 h using a UV/Visible Ultrospec 2000 spectrophotometer (Pharmacia Biotech, Cambridge, England).

To measure copper resistance, $CuSO_4$ (0 – 4.8 mM end concentrations) was added to the growth medium. A 96-well microplate with the samples was incubated for 21 h at 30°C. The OD_{600} was measured every 15 min with a SpectraMax spectrophotometer (Molecular Devices, Wokingham, UK). The minimum inihibitory concentration (MIC)

of copper was determined as the concentration of $CuSO_4$ (g/L) that did not result in visible growth after 15 hours of incubation.

Pilin overexpression in *L. lactis*

The *spaCBA-srtC1-srtC2* locus (designated as *pil* locus) including its 300-bp upstream region was amplified with KOD Hot Start Polymerase (Merck Millipore, Madison, Wisconsin, USA) using the pilinPstI forward and the pilinXhoI reverse primers (S1 Table). The purified PCR product was digested with PstI and XhoI and ligated to similarly digested pIL253 using T4 DNA Ligase (Invitrogen, Breda, The Netherlands). The ligation mixture was used to transform (61) electro-competent (62) MG1363 cells. Transformants harbouring the anticipated pIL253*pil* plasmid (Table 1) were identified performing colony PCR with primers pSH74_FW and pSH74_RV (S1 Table).

An internal deletion of 1451 bp in the *spaCB* gene was constructed by digestion of pIL253*pil* with AatII followed by re-ligation and introduction of the plasmid in *L. lactis* MG1363. The resulting plasmid was designated pIL253*pilΔ1.* Transformation of plasmids into other strains was performed with the protocol described above.

Cell aggregation

L. lactis cells from a 10-ml overnight culture were washed twice with 10 mL sterile 10 mM phosphate buffer (pH 6.8) and re-suspended in the same buffer. Cell sedimentation was observed visually and cell chaining was examined using bright field microscopy.

Scanning Electron Microscopy (SEM)

Bacterial cells were cultured for 1 day on GM17 agar plates. From plates with 50-100 colonies, small pieces of agar gel carrying less than 5 colonies were cut out and placed in a microscope sample holder. All further steps of cell fixation, washing, dehydration, staining, freeze-drying, electron microscopy, and image analysis were performed according to (63). A FEI Magellan 400 FESEM electron microscope (Wageningen Electron Microscopy Centre, The Netherlands) was used for imaging.

Conjugation

Conjugation experiment were performed as described previously (64, 65) with *L. lactis* MG1614 as the recipient strain. Transconjugants were selected on milk agar plates containing 0,004 % bromocresol purple (Merck, Darmstadt, Germany), streptomycin (100 µg/mL) and rifampicin (50 µg/mL) with the donor strains NCDO712(pIL253*pil*) or MG1299(pIL253*pil*). LM17 plates supplemented with streptomycin (100 µg/mL), rifampicin (50 µg/mL) and 1.2 mM $CuSO_4$ were used for the conjugative transfer of pNZ712, carrying the copper resistance genes, from *L. lactis* NCDO712 to *L. lactis* MG1614.

Results and Discussion

Chromosomal differences between *L. lactis* strains NCDO712 and MG1363

Sequencing of the total DNA of NCDO712 allowed detecting 11 Single Nucleotide Polymorphisms (SNPs) between the chromosomes of *L. lactis* NCDO712 and its plasmid-cured derivative MG1363 (66). The latter was isolated in 1983 following multiple rounds of chemical- and protoplast-induced plasmid curing (26). Among the 11 SNPs in the chromosome of *L. lactis* NCDO712, three are synonymous and three are in intergenic regions, while the other five lead to amino acid changes in proteins (Table S2). The nucleotide sequencing data also suggests the occurrence of genome re-arrangements mainly due to mobile genetic elements, but their verification was outside the scope of this study.

Only one of the three SNPs in the intergenic regions is predicted to be in a promoter region, that of the *mtlA* gene encoding a putative mannitol-specific PTS system EIIBC component. Differential RNA sequencing has pinpointed the transcription start site (TSS) of *mtlA* at position 26465 (67). The mutation at the position 26455 suggests that the -10 box is altered from an optimal TATAAT into TACAAT. Furthermore, three of the protein sequence-affecting SNPs in the genes encoding a hypothetical protein and two transposases were predicted not to affect protein functions. These predictions were made using SIFT, an algorithm that analyzes the effect of mutations based on the degree of conservation of amino acid residues (60, 68). Mutations in the *gapB* and *tsf* genes encoding the glyceraldehyde 3-phosphate dehydrogenase and elongation factor TS, respectively, were predicted to affect protein function. Whether these mutations are caused by genetic drift or whether they might confer a fitness advantage in a laboratory environment is unclear. However, the data indicate that the number of SNPs that accumulated since the isolation of MG1363 more than 30 years ago is limited.

MG1363 is also described to have lost a ≈40 kb DNA fragment during its generation from NCDO712, which rendered MG1363 insensitive to UV induced lysis (69). Based on this information we expected to find this additional ≈40 kb fragment in the genome

of NCDO712 when comparing the sequence to MG1363. Because we were not able to identify such a fragment we tried to induce lysis in NCDO712 by treatment with either mitomycin C or exposure to UV radiation. With neither approach we were able to induce lysis in NCDO712, suggesting that the variant we were working with did spontaneously loose this fragment. A NCDO712 variant without this 40 kb fragment is phenotypically the same as strain SH4109. However due to potential genome rearrangements in our NCDO712 isolate and the known high genome plasticity in this strain and its derivatives, it cannot be concluded that our isolate is identical to SH4109.

L. lactis NCDO712 harbors six plasmids

Assembly of all nucleotide sequence reads that did not map onto the chromosome of *L. lactis* MG1363 revealed that *L. lactis* NCDO712 carries a total of 6 rather than the previously described 5 plasmids (26). Using *L. lactis* NCDO712 derivatives harboring single plasmid species (26), we linked the plasmids identified here to the earlier described plasmids of this strain (Table 2). The published plasmid sizes did not fully correspond to the respective sizes determined here. This could be caused by the limitations of size estimation based on agarose gel electrophoresis used previously (26). However, we cannot exclude that plasmid rearrangements have occurred during strain propagation over the years that could account for (part of) the differences, although we did not observe such genetic changes in the 55.4-kb pLP712 plasmid (see below) (27). The additional plasmid identified in this study, designated pNZ712, has a size similar to that of pLP712, which may explain why it escaped detection in 1983 (26).

Table 2. Comparison of NCDO712 plasmids with earlier studies.

Plasmids described in (26)		Plasmids analyzed in this study			
Plasmid	**Size**[1]	**Plasmid**	**Size**	**Plasmid copy number/mean coverage**[2]	**Replication mode**
pLP712	33 MDa, ≈50 kb	pLP712	55 395 bp	2 (423)	Theta
pSH71[3]	1,8 MDa, ≈3 kb	pSH71	2 062 bp	3 (673)	RCR
pSH72[4]	2,5 MDa, ≈4 kb	pSH72	3 597 bp	4 (921)	Theta
pSH73[5]	5,2 MDa, ≈8 kb	pSH73	8 663 bp	3 (674)	Theta
pSH74	9 MDa, ≈14 kb	pSH74	15 518 bp	3 (697)	Theta
-	-	pNZ712	49 832 bp	2 (471)	Theta

[1] Plasmid size in original publication is given in MDa (1MDa ds-DNA = 1.52 kb (https://tools.thermofisher.com).

[2]Estimated on the bases sequence coverage in comparison to that of the chromosomal DNA. Coverage number is based on the analysis of 6 million sequence reads. The mean chromosomal DNA coverage in the same analysis was 198.

[3] pSH71 in this study differs at 6 positions from the publically available sequence of pSH71 (NCBI accession number A09339; de Vos W.M., 1987): T712-deleted, T713-deleted, A731-deleted, G803A, T1234-inserted, C1414-deleted (the nucleotide before the position number indicates the sequence in the plasmid sequenced here, the description after the position number indicates the sequence in accession number A09339).

[4] pSH72 differs by 3 bp from pND324 (70) (NCBI reference sequence: NC_008436.1): T1295G, G1384A and C3349-deleted. pSH72 is 99% identical to pND324

[5]pSH73 is 100% identical to pAG6 (NCBI acession number: AB198069, GI: 70067197)

The copy number of the individual plasmids varied between 2 and 4, based on mean coverage number of chromosomal DNA and plasmid coverage (Table 2). The plasmid replication mode was determined using previously described criteria (2, 53). Rolling circle replication (RCR) was identified on the basis of the presence of Rep-family protein encoding genes and a double-stranded origin (*dso*) of replication, while a replication initiator protein-encoding *repB* gene and an origin of replication (*ori*) are

indicative of theta-type plasmid replication (9). These analyses indicate that pSH71 replicates through a rolling circle mechanism (9, 45), while the other 5 plasmids replicate using a theta-type mechanism (9).

Plasmids pLP712 and pSH71 have been sequenced and described earlier (27, 45). The nucleotide sequence of the pLP712 is identical to the one determined here, except for a single nucleotide difference, whereas the pSH71 sequence differs by 6 nucleotides (Table 2).

To investigate the relationships of plasmids pSH72, pSH73, pNZ712, and pSH74 with other known plasmids we compared their nucleotide sequences with 1955 plasmid sequences in the NCBI database (database consulted on February 1, 2015). pSH72 (3597 bp) has the highest copy number, approximately 4 copies per cell, and only appears to encode the replication genes *repB, repX,* and *repC*. Except for 3 nucleotide differences pSH72 is identical to plasmid pND324 isolated from *L. lactis* subsp*. lactis* LL57-1 (70) (Table 2). The biological function of this plasmid is unclear. pSH73 is identical to pAG6, a plasmid isolated from *Lactococcus lactis* ssp*. cremoris* 712, which is most likely the same strain as NCDO712 or a derivative of it (29). The only SNP (A → G, pAG6 → pSH73) is at nucleotide position 1143 of the *hsdS* gene encoding a type-I restriction/modification system specificity subunit. This SNP is silent and does not change the amino acid sequence. Plasmid pSH73 harbors, next to the replication genes *repX* and *repB*, also *cadCA* genes that are predicted to encode a cadmium resistance regulatory protein and a cadmium efflux ATPase (Fig. 1 and Table S3).

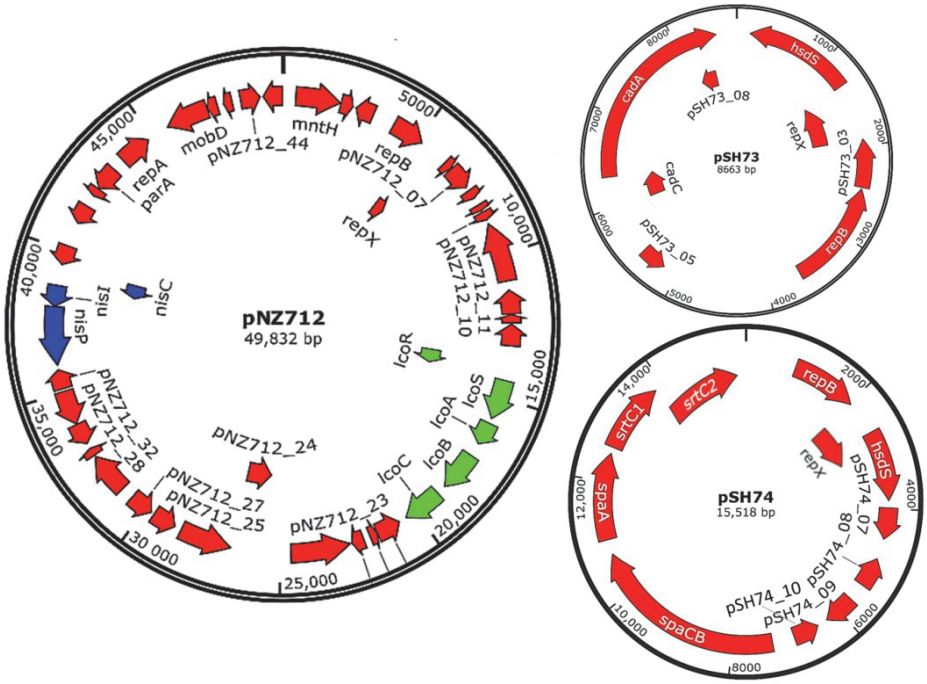

Figure 1. Plasmid maps of pSH73, pSH74, and pNZ712. Plasmid pNZ712 includes genes encoding functional nisin immunity (*nisCIP*) and copper resistance (*lcoRSABC*). The 16-kb plasmid pSH74 contains a novel 8-kb pilus gene cluster *spaCB-spaA-srtC1-srtC2*. Plasmid pSH73 harbors *repX*, *repB* and *cadCA* genes. The latter genes were annotated as a cadmium resistance regulatory protein and a cadmium efflux ATPase.

The two other plasmids pSH74 and pNZ712 have partial similarity (93-99% nucleotide identity) to known *L. lactis* plasmids. These high identities were observed in genes encoding several functions such as replication, transposase, resolvase, and in copper resistance-associated genes and in the *nisCIP* genes (Fig. 1 and Fig. S1, Table S4). Detailed sequence analysis of pSH74 identified putative pilus biosynthesis genes, which we annotated as *spaCB-spaA-srtC1-srtC2*.

Plasmid pNZ712 specifies nisin immunity and copper resistance

Nisin is a lanthionine-containing antimicrobial peptide that binds to lipid II, disrupts the cytoplasmic membrane and causes death of susceptible bacterial cells (71). The nisin operon *nisABTCIPRKFEG* carries, next to the nisin structural gene *nisA*, genes responsible for nisin modification, transport and precursor cleavage, genes involved in nisin operon regulation and genes specifying resistance to the bacteriocin (47, 72). Plasmid pNZ712 carries *nisCIP,* albeit *nisC* is only partially present. The *nisC* gene product NisC, in concerted action with NisB, is involved in posttranslational modification of the nisin precursor. The *nisI* gene specifies nisin immunity, while *nisP* encodes the serine protease involved in maturation of the nisin precursor.

To determine whether pNZ712 *nisI* is functional, *L. lactis* NCDO712 was grown in LM17 medium supplemented with 0 or 20 ng/mL nisin. *L. lactis* NZ9700 was used as a nisin resistant control. All strains reached a maximal OD_{600} of 2.96±0.63 after 7 h of growth without nisin. When they were grown in the presence of 20 ng/mL nisin, the positive control strain NZ9700 reached an OD_{600} of 1.8±0.06. *L. lactis* NCDO712 and SH4109, two strains carrying all 6 plasmids including pNZ712 (*nisCIP*), reached an OD_{600} of 0.63±0.01 and 0.67±0.09, respectively. The latter strains reached lower final optical densities than strain NZ9700, which has full immunity function as it carries *nisFEG* genes encoding an ABC transporter (73) that contributes to nisin immunity *nisI* in NCDO712. The plasmid-free *L. lactis* MG1363 and other derivatives carrying single plasmids from *L. lactis* NCDO712, but not pNZ712, reached an OD_{600} of only 0.06±0.046. From these results we conclude that the pNZ712-encoded *nisI* is functional and provides nisin resistance. The production of nisin has been suggested to give an advantage to *L. lactis* in a plant environment where *nis* genes often co-occur with sucrose utilization genes (74). The nisin operon seems less prevalent in dairy isolates, indicating a limited benefit in this environment. The constitution of the starter culture from which *L. lactis* NCDO712 was isolated is unknown, but if this culture would have contained a nisin producer the maintenance of the *nisCIP* genes could have given an advantage.

Plasmid pNZ712 also carries a set of genes, *lcoRSABC*, which may be involved in copper resistance. Plasmid-encoded copper resistance has been described previously in *Streptococcus* (now *Lactococcus*) *lactis* (75, 76), but has not been described for *L. lactis* NCDO712. The genes *lcoRS* are involved in transcription regulation of *lcoABC,* the products of which confer copper resistance by lowering the accumulation of copper inside the lactococcal cell (7). Furthermore, pNZ712_*23* encodes a putative copper-(or silver)-translocating P-type ATPase.

To examine whether pNZ712 specifies copper resistance, *L. lactis* NCDO712 and several of its derivatives were grown with or without $CuSO_4$. *L. lactis* MG1363 and other NCDO712 derivatives did not grow when 0.8 mM or more $CuSO_4$ was present in the medium. *L. lactis* NCDO712 was the only of the tested strains that was able to grow in the presence of up to 4 mM (1 g/L) $CuSO_4$, indicating that the *lcoRSABC* gene cluster and/or pNZ712_*23* on pNZ712 are functional. The minimum inhibitory concentration of $CuSO_4$ was 1 g/L (Fig. S2). We also showed that pNZ712 harboring the copper- and nisin-resistance genes can be transferred from NCDO712 to MG1614 via conjugation using copper as a selective marker. The conjugation frequencies were 8.1e-8 ± 2.6e-8 transconjugants per donor cell and using PCR we confirmed that only pNZ712 was transferred to the recipient strain.

Interestingly, a recently diverged derivative of *L. lactis* NCDO712, strain C2 (69, 77), was reported to carry 5 plasmids. The resistance of this strain to copper and other metal ions was suggested to be encoded by the lactose plasmid pLM3001 (approximately 30 MDa), of which the nucleotide sequence has not been determined (75). Our results, showing that copper resistance and lactose utilization genes reside on different plasmids in strain NCDO712, indicate that plasmid re-arrangements may have occurred in either or both of these strains (77).

A novel pilus gene cluster is present on pSH74

Plasmid pSH74 harbors an 8-kb gene cluster, which we annotated as *spaCB-spaA-srtC1-srtC2* (Fig. 2). The order of genes in pilus biosynthesis clusters differs among gram-positive bacteria (39, 78). The synteny on pSH74 resembles that of the *spaC-spaB-spaA-srtC1* cluster of *Lactobacillus rhamnosus* GG most (42, 43, 79). However, *spaC* and *spaB* seem to be fused in *L. lactis* NCDO712 (see below) and the operon-encoded proteins display only 30 to 45 % amino acid sequence identity. An additional difference to *L. rhamnosus* GG is that the GG pilus cluster contains only one sortase gene while the pSH74 pilin gene cluster contains two adjacent *srtC* genes encoding SrtC enzymes of 413 and 392 amino acid residues, respectively. These show only 38% mutual amino acid identity. Two or even three consecutive *srtC* genes have previously been identified in the pili biosynthesis gene clusters in *Streptococcus agalactiae, S. pneumoniae,* and *Clostridium diphteriae* (80, 81).

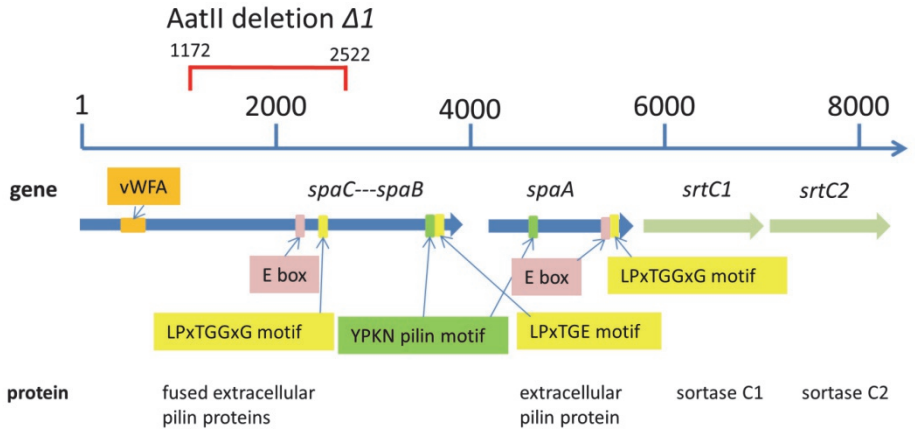

Figure 2. Schematic representation of the pilus gene cluster on pSH74 and the suggested functions of its constituting genes (not to scale). The internal deletion of 1451 bases in *spaCB* is indicated in red.

The GC content in the NCDO712 *pil* operon (35.3%) closely matches that of the NCDO712 chromosome (35.7%) suggesting that its acquisition is not a recent event or that it has been transferred from a microbial species with a similar GC content. The latter is consistent with the presence of a highly homologous pilus gene cluster in the genome of *Leuconostoc citreum* (>90% protein sequence identity) with a GC content of 38.9%.

Previously two other pilus gene clusters were identified in *L. lactis*. Oxaran and co-workers (22) identified a chromosomally encoded pilus gene cluster in *L. lactis* IL1403, *yhgD, yhgE, yhhA, yhhB*. This chromosomal cluster, which does not show homology at the protein or synteny level to the pSH74 pilus gene cluster, appears to be present in all lactococcal genomes known to date, including those of *L. lactis* strains SK11, KF147, and MG1363 (*llmg_1800-1803*) (22) as well as NCDO712. Similarly, a plasmid-located 6.9-kb pilus gene cluster *yhgE2-srtC2-yhhB2-ORF4* detected in *L. lactis* TIL448 (21) does not show significant homology or synteny to the pilus gene cluster identified here. A sequence comparison between the chromosomal pilus gene cluster in NCDO712 and the plasmid pSH74-located gene cluster identified here showed no synteny. Protein sequence analysis showed that SrtC1 and SrtC2 have a 35% (over 66% of the query) and 32% (over 73% of the query) identity with the chromosomally encoded SrtC (*llmg_1801*), respectively. Typically less than 30% amino acid identity is observed for the other proteins if the query sequence is at least 20% of the length of the protein. The presence of two pilus gene clusters in one strain suggests that they might have different functions and it raises the question whether *L. lactis* NCDO712 is able to produce the two types of pili simultaneously. Previous work has shown that *L. pneumophila* AA100 expressed both, long and short pili, but not at the same time (82) while concurrent expression of two types of pili has been reported for *E. faecium* (83). *L. lactis* MG1363, which contains the chromosomal *pil* operon, only produces pili on its cell surface when it overexpresses the *pil* operon from pSH74, as is clear from the electron microscopy data and phenotypic characterization presented here. Whether conditions exist in which both types of pili are expressed simultaneously in *L. lactis* NCDO712 remains to be determined.

We hypothesize that the functions of the pilin gene products of *L. lactis* NCDO712 pSH74 are similar to those in *Lb. rhamnosus GG* (80) and other gram-positive bacteria (36, 38, 84, 85). Based on this hypothesis the SpaA protein of *L. lactis* NCDO712, $SpaA^{LL}$, is presumably the major pilus backbone subunit (Fig. 2). It contains the typical C-terminal LPSTGGAG motif involved in sortase C-catalyzed transpeptidation and has the characteristic YPKN "pilin motif". The YPKN motif is involved in intermolecular peptide bond formation between the carbonyl-group carbon of the threonine residue of the pilin subunit and the side-chain of the lysine in the pilin motif of the neighboring pilin subunit (41). These bonds lead to the formation of membrane-associated covalently-linked dimers with a pilin motif that can interact with other pilin subunits, forming an elongated pilus fibre (41). $SpaA^{LL}$ also carries the YVLNETKAP "E box", which has a structural role in pilus assembly (52). This was illustrated by the involvement of the "E box" of SpaA from *Corynebacterium diphtheria* in the attachment of SpaB to polymerized SpaA fibres (38).

Lb. rhamnosus GG SpaB ($SpaB^{GG}$) is the basal pilin subunit and $SpaC^{GG}$ is the pilin tip protein. While *spaC* and *spaB* are separate genes in *Lb. rhamnosus* GG, they are fused in *L. lactis* NCDO712. The first 840 amino acid residues of *L. lactis* NCDO712 SpaCB ($SpaCB^{LL}$) correspond to $SpaC^{LL}$, while the remaining 260 residues resemble $SpaB^{LL}$. The SpaC segment in $SpaCB^{LL}$ contains an LPSTGGSG motif which is a potential cleavage site for SrtC between the threonine (T) and glycine (G) residue (41), thereby splitting $SpaCB^{LL}$ into the respective proteins, $SpaC^{LL}$ and $SpaB^{LL}$. The $SpaB^{LL}$ resembling part of $SpaCB^{LL}$ contains a C-terminal LPDTGE motif (Fig. 2, B) that is predicted to be targeted by SrtA and serve as a peptidoglycan anchoring sequence (79, 80). Hence, $SpaB^{LL}$ is most likely at the base of the lactococcal pilus.

The SpaC^{LJ} segment of SpaCB^{LJ} has an "E box" (YALTETKTP), which has a structural role in pilus assembly (14, 61, 78). Possibly, after cleavage of SpaCB^{LJ} by SrtC, the "E box" is used to link SpaC^{LJ} to SpaA^{LJ}. The SpaC^{LJ} segment contains a von Willebrand type-A domain (vWFA). It has been speculated that a vWFA domain, which is also present in SpaC of *L. rhamnosus* GG (43), may have lectin-like binding properties and could bind to sugars with high carbohydrate specificity. SpaC^{LJ} also carries a collagen-binding domain and two collagen-binding surface-protein-Cna B-type domains, which might be involved in bacterial adhesion to surfaces (IPR008970; SSF49478) (http://supfam.org). Taken together, these observations suggest that, analogous to SpaC^{GG} in the *Lb. rhamnosus* GG pilus, SpaC^{LJ} might fulfill the tip protein function.

Based on protein homology searches we identified similar pilus gene clusters in *L. lactis* subsp. *cremoris* CNCM I-1631 contig_071 (accession number: AGHX01000000; LLCRE1631_01806, LLCRE1631_01807, LLCRE1631_01808, LLCRE1631_01809) (97% identity), in *L. lactis* subsp. *lactis* 1AA59 contig_056 (accession number: AZQT01000047.1 and AZQT01000000; KHE76387.1, KHE76388.1, KHE76389.1, KHE76390.1) (100% identity), as well as in the *Leuconostoc citreum* genome (accession numbers: WP_048699698, WP_048699696, WP_048699695, WP_048699693) (>90% identity). In all these cases, the corresponding protein sequences are annotated as encoding hypothetical proteins. The *spaCB* genes are fused in these strains as well as in *L. lactis* NCDO712, suggesting that this might be of relevance for e.g., controlling the ratio of the two proteins in the pilus structure.

Pilus formation and attachment to peptidoglycan in *Lb. rhamnosus GG* is governed by the pilin-specific sortase SrtC1 and the house-keeping sortase SrtA. SrtC1 specifically targets a triple glycine motif LPxTGGxG at the N-terminal end of pilin proteins, and catalyzes their assembly into pili (84). The chromosomally-encoded enzyme SrtA targets N-terminal LPxTGE or LPxTGD motifs (80) and covalently anchors extracellular proteins, including pilin proteins, to peptidoglycan in the cell wall. The SrtA recognition sequence does not necessarily prevent its recognition by a sortase other than SrtA (80). Thus, SrtA and SrtC1 may each be able to recognize both LPxTGD/E and LPxTGGxG motifs used for pilin polymerization and anchoring (80).

However, the protein-structural features involved in motif recognition by sortases are currently unknown. When *spaA* and *srtC1* of *Lb. rhamnosus GG* were co-expressed in *L. lactis* NZ9000, it was observed that SrtC1 recognized and polymerized the SpaA protein. *Lb. rhamnosus GG* SpaC has the same LPxTG motif as SpaA (80), therefore, SrtC1 possibly recognizes the pilus tip protein SpaC similar to SpaA, while SrtA only anchored SpaB to the peptidoglycan. Based on the similarity of the pSH74-located pilus cluster to that of *Lb. rhamnosus* GG, we speculate the gene functions to be similar, namely that one or both of the plasmid encoded C sortases in *L. lactis* NCDO712 are responsible for the assembly of pilin proteins in this strain, while the chromosomally encoded SrtA (llmg_1449) covalently anchors the pili to the peptidoglycan of the cell wall (79, 80). Data obtained from RNAseq performed on total RNA isolated from *L. lactis* NCDO712 growing under different stress conditions revealed that *srtC1* and *srtC2* are usually co-expressed, while there is no correlation in expression levels between the other genes of this gene cluster (67).

The pilus gene cluster on *L. lactis* NCDO712 pSH74 is functional

To examine whether the identified putative pilin genes are functional, the entire pilus gene cluster with its native promoter was cloned in the medium-copy number (45-85) plasmid pIL253 (52), resulting in pIL253*pil*. Initial attempts to clone *pil* downstream of the nisin-inducible *nisA* promoter or the constitutive *purC* promoter failed. The few clones that were obtained all carried the same internal deletion in the *spaCB* genes. Deliberate deletion of a similar internal 1.5 kb fragment resulted in pIL253*pilΔ1* (Table 1, Fig. 2). The deletion leads to a frame-shift mutation shortening the SpaCB[U] protein by 744 amino acid residues and resulting in a 394-residue truncated SpaC protein. This protein still carries the vWFA domain but the YPKN pilin motif, the LPDTGE motif (Fig. 2), the"E box" and LPS amino acids of LPSTGGSG motif were deleted. RNAseq data revealed that the native promoter includes a leader sequence of ≈200 nt upstream of *spaCB* (67). Although the presence of this leader seems important for the successful overexpression, its precise role is currently not known. Both plasmids pIL253*pil* and pIL253*pilΔ1* were introduced in *L. lactis* strains MG1363 and IL1403.

The observed pilus overexpression from the pIL253-based vector resulted in cell aggregation and rapid sedimentation of the cultures of both strains (Fig. 3). The cells also grew in chains containing more than 10 cells, while the wild type strain MG1363 and control strain MG1363(pIL253) did not form such chains (Fig. 4). Cells expressing the truncated version of SpaCB^{U} displayed neither cell aggregation (Fig. 3) nor cell chaining (Fig. 4). As the anchoring of pili to the cell surface is expected to be essential for their functionality the effect of the *spaCB* truncation is consistent with the loss of cell aggregation and chaining.

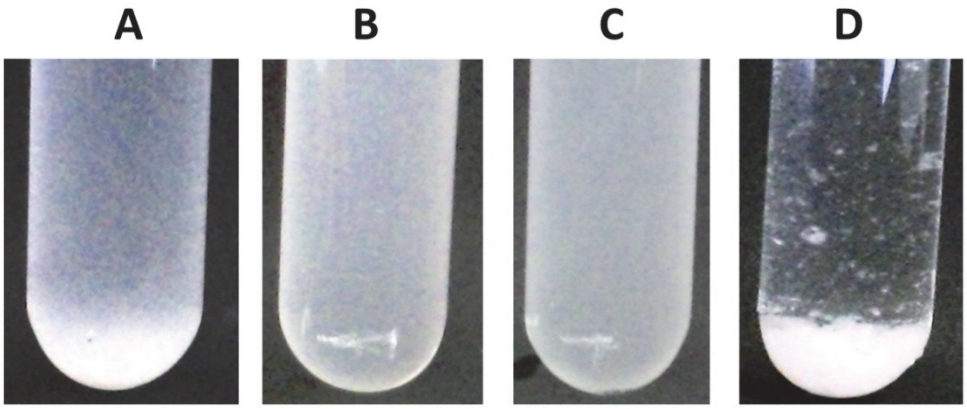

Figure 3. Cell aggregation after pilus gene overexpression. A – *L. lactis* NCDO712, B – empty vector control MG1363(pIL253), C – MG1363(pIL253*pilA1*), D – MG1363(pIL253*pil*). The images were taken 3 min after re-suspension of cells from an overnight culture.

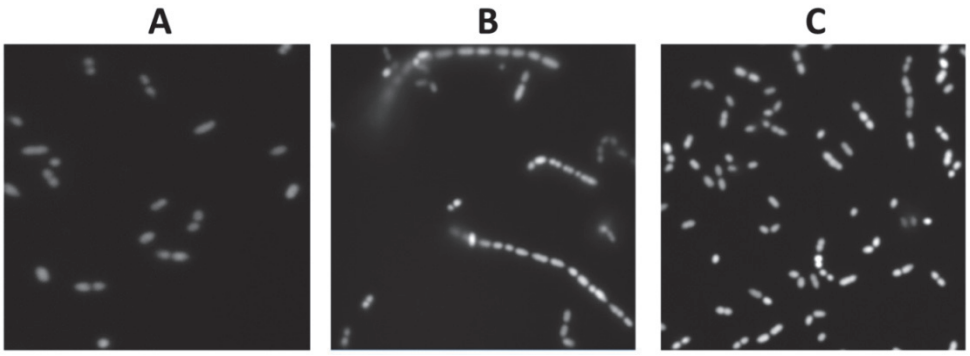

Figure 4. Pilin gene overexpression in *L. lactis* MG1363 leads to increased chain length. A – MG1363(pIL253), B – MG1363(pIL253*pil*), C – MG1363(pIL253*pilΔ1*).

Scanning electron microscopy (SEM) revealed that *L. lactis* NCDO712 (Fig. 5, A) cells have a relatively rough surface while those of *L. lactis* IL1403 were very smooth (compare A and B in Fig. 5). The introduction of pIL253*pil* resulted in an increased roughness of the cell surface for both IL1403 and MG1363. Furthermore, even though *L. lactis* NCDO712 harbors the *pil* operon on pSH74, pili were not visible on the surface of these bacteria. This indicates that expression of pili from the native plasmid, estimated to be present at 3 copies per cell (Table 2), is not high enough to allow their detection. However, expression of the same operon (with the native promoter) from plasmid pIL253, which is present in higher (45-85) copy numbers (52), is sufficient to detect pilus-like structures on cells of *L. lactis* IL1403(pIL253*pil*) (Fig. 5, C and Fig. 6, E), MG1363(pIL253*pil*) (Fig. 5, E and Fig. 6, C) and NCDO712(pIL253*pil*) (Fig. 6, A). Similar to what we found in NCDO712, detection of pili from the *L. lactis* IL1403 chromosomal pilus gene cluster *yhgD, yhgE*, *yhhA*, *yhhB* could only be achieved by their overexpression (22). However, in *L. lactis* isolates from vegetables, such as KF282 and NCDO2118, and in the clinical isolates 2885-86 and 810-85, pili were detectable under standard growth conditions using negative staining and TEM analysis (22). In another study wild-type *L. lactis* TIL448 was shown to carry a plasmid-encoded 6.9-kb pilus gene cluster, *yhgE2-srtC2-yhhB2-ORF4*, that lead to the formation of short (260-440 nm) and low density pili at the cell surface detectable with AFM (21).

Overexpression of the *L. lactis* TIL448 pilus gene cluster *yhgE2-srtC2-yhhB2-ORF4* in *L lactis* MG1363 (TIL1293) led to thicker (2.3 nm), longer (1.2-2.5 μm) and higher density pili at the cell surface (21).

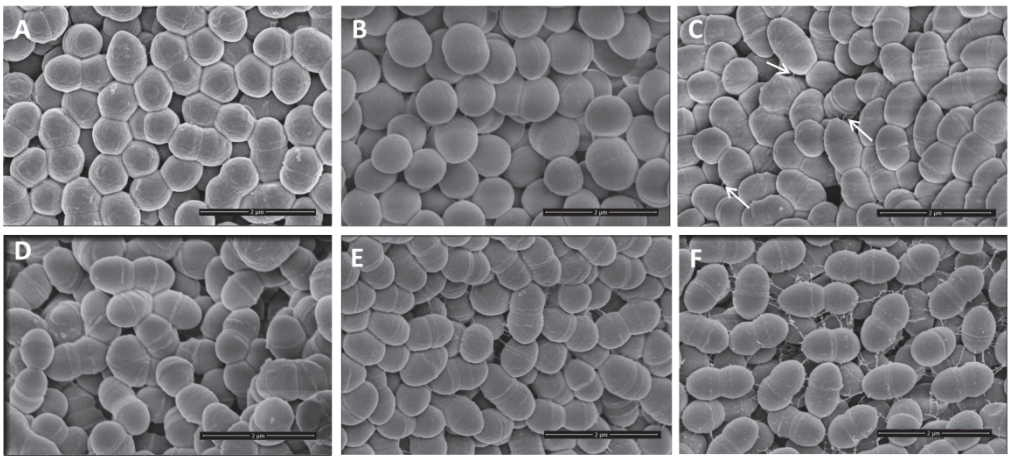

Figure 5. Scanning electron microscopy at 50.000 x magnification of *L. lactis* strains overexpressing *spaCB-spaA-srtC1-srtC2*. A - NCDO712, B - IL1403, C – IL1403(pIL253*pil*), D - MG1363(pIL253), E – MG1363(pIL253*pil*), F –MG1363(pIL253*pilΔ1*). White arrows indicate pili in panel C. Black bars: 2 μm in all panels.

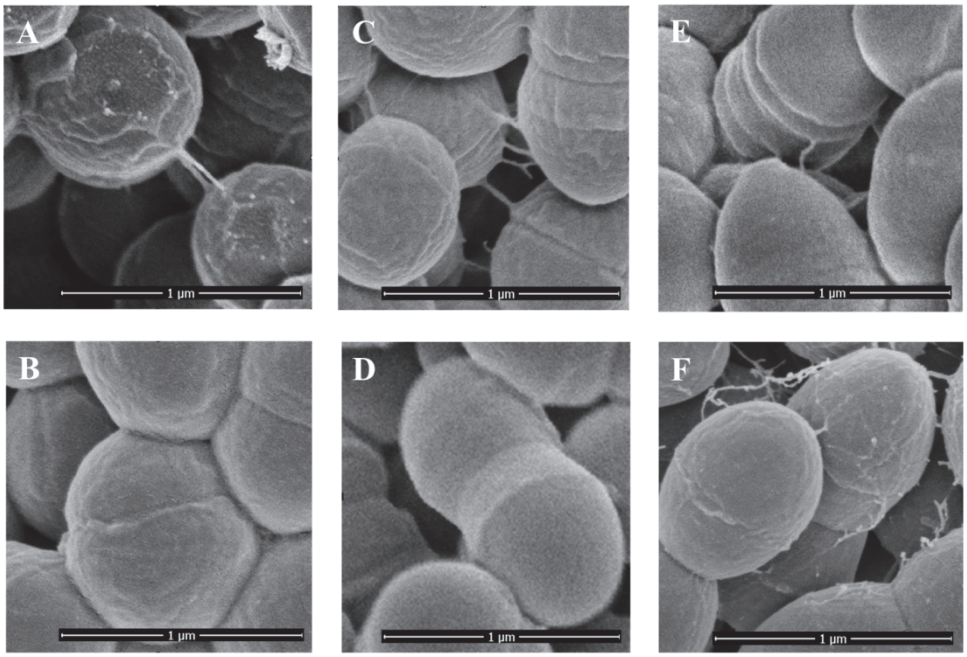

Figure 6. Scanning electron microscopy of *spaCB-spaA-srtC1-srtC2* overexpressing *L. lactis* strains. A - NCDO712(pIL253*pil*), B – NCDO712, C – MG1363(pIL253*pil*), D - MG1363(pIL253), E - IL1403(pIL253*pil*), F- MG1363(pIL253*pilΔ1).* Black bars: 1 µm in all panels.

Interestingly, MG1363(pIL253*pilΔ1*) cells expressing the truncated version of SpaCB^{LI} (Fig. 2, B; Fig. 5, F) were also decorated with pilus-like structures, but these appeared to be more disoriented (Fig. 5, compare E and F), which would be consistent with SpaA being the shaft pilin. This disorientation may imply that the pili produced by MG1363(pIL253*pilΔ1*) are not appropriately attached to the peptidoglycan, which is consistent with the truncated form of SpaCB^{LI} lacking the predicted SrtA motif for peptidoglycan anchoring. All in all, the results reveal that the *spaCB-spaA-srtC1-srtC2* pilus gene cluster from pSH74 of *L. lactis* NCDO712 is functional and leads to the formation of pilus-like appendices on the surface of lactococcal cells.

The *Lactobacillus rhamnosus* GG pilus operon *spaC-spaB-spaA-srtC1* is involved in attachment to human epithelial colorectal adenocarcinoma cells (43), and an intestinal mucus-binding capacity was attributed to the tip pilin subunit SpaC (42) via mucus-binding domain. *Lb. rhamnosus GG* also carries a pilus gene cluster, *spaFED,* on its chromosome, where SpaD is the pilus backbone protein, SpaE is at the base of the pilus structure, and SpaF is the minor pilin subunit that locates to the tip of the pilus. SpaF was also shown to be responsible for adhesion of pili-carrying cells to the intestinal mucus (42, 88). Furthermore, cloning of $spaC^{GG}$, $spaB^{GG}$, $spaD^{GG}$, $spaE^{GG}$, $spaF^{GG}$ in *Escherichia coli* and assessment of the adherence of these proteins to human intestinal mucus reveled that also the SpaB pilin subunit plays a role in binding to intestinal mucus, through electrostatic contacts (42). In the *L. lactis* TIL448 pilus gene cluster *yhgE2-srtC2-yhhB2-ORF4*, the latter is the putative pilus tip protein. It contains a lectin-like domain (PF00139) predicted to have carbohydrate-binding properties, which could be involved in binding to plant cell walls (21). Furthermore, overexpression of TIL448 *yhgE2-srtC2-yhhB2-ORF4* in *L. lactis* MG1363 (TIL1293) was shown to increase attachment of the bacteria to Caco-2 cells (21). No significant differences in attachment to Caco-2 and HT29 (a human colonic carcinoma cell line) cells were observed when stationary-phase cells of *L. lactis* with or without pIL253*pil* were tested (Table S5). Similar to our results earlier studies also described that strain NCDO712 (synonym C2 or TIL256) did not adhere to Caco-2 cells (21).

In gram-negative bacteria the length of pili is approximately 1-2 μm, and the diameter is between 1 nm and 10 nm (36, 41). In gram-positive bacteria pili are more varied with reported lengths of 0.3-3 μm, and a diameter between 3-10 nm (41). The *Lb. rhamnosus* GG *spaC-spaB-spaA-srtC1* pili are up to 1 μm in length, with a diameter of 1 to10 nm (43, 79). The pili encoded by *yhgD, yhgE, yhhA, yhhB* in *L. lactis* IL1403 are up to 3 μm long and have a diameter of approximately 5 nm (22). Pili on the surface of wild type *L. lactis* TIL448 specified by *yhgE2-srtC2-yhhB2-ORF4* were short (up to 450 nm) and thin, but the pili of the overexpressing *yhgE2-srtC2-yhhB2-ORF4* cluster strain *L. lactis* MG1363 (TIL1293) were longer with an average length of 2 μm and diameter of approximately 2.3 nm (21). Interestingly, the pili overexpressed by

MG1363(pIL253*pil*) are shorter and thicker than earlier reported lactococcal pili (21, 22, 24), having a length of 200-240 nm and a diameter between 18 and 20 nm. The appendages produced by MG1363(pIL253*pilΔ1*) were thinner and had an average diameter of approximately 14 nm and the length of 120-560 nm.

Conjugation efficiency

One of the roles of certain pili in gram-negative bacteria is to enable conjugation (89). The overexpression of the pili identified here frequently displayed cell-cell contact (Fig. 6, A and 6, C), which led us to examine whether the *L. lactis* NCDO712 *pil* operon might play a role in DNA transfer by conjugation. *L. lactis* NCDO712 and MG1363 carry a 50-kb sex factor in its chromosome that is involved in co-integrate formation with and subsequent conjugal transfer of the lactose/protease plasmid pLP712 (29, 35). This process can be readily quantified using the plasmid-free, lactose-deficient *L. lactis* MG1614 (Lac^-, $PrtP^-$, $Strep^R$, Rif^R) (Table 1) as a recipient and selecting for lactose-fermenting colonies that are also resistant to rifampicin and streptomycin. The efficiency of transfer of pLP712 from strains NCDO712 and MG1299 was up to 22 and 16 fold increased, respectively, when the strains carried pIL253*pil* and, thus overexpressed pili (Table S6). We did not observe co-transformation of pIL253*pil* during the conjugation of pLP712. Although the observed increase in the frequency of conjugation is limited, the observations indicate that the pili contribute to the efficacy of exchange of DNA between lactococcal cells. Whether this effect is caused by pili-mediated cell clumping and, with that, increased cell-cell contact (Fig. 6), or whether the pili are involved in actual DNA transfer requires further investigation.

Conclusion

Surface decoration of bacterial cells with pilus-like structures is widespread and functions such as DNA transfer (37), surface attachment and biofilm formation (22) or interaction with host cells (21) were described (36). Despite the increasing number of examples of pilus gene clusters in lactococci it is still unclear what the functions of the encoded pili are. In lactococcal strains isolated from plant material or in human isolates, pilus expression was shown to occur under standard laboratory conditions (22), but this was not observed for any of the investigated dairy isolates (1, 7, 25). Pili of plant associated *Pseudomonas syringae* were described to be involved in the attachment of the bacteria to the plant surface (44). It remains to be determined whether pili in dairy lactococci are evolutionary remnants of their proposed plant ancestry (90) or whether they fulfill a function in the dairy environment.

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Supporting information for Chapter 2

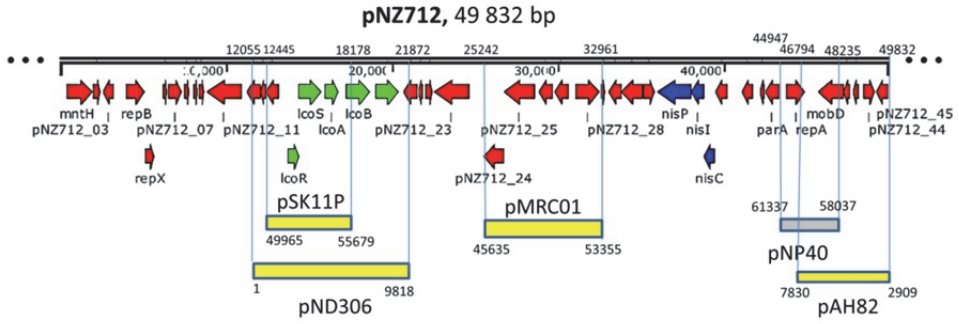

Figure S1. Plasmid identity to pNZ712. Five out of 100 plasmids with high partial sequence identity to pNZ712 are shown. The similar regions include genes encoding copper resistance genes *lcoRSAB*, mobilization genes *mobD, mobC* and a gene involved in replication (*repA)* with 99% (yellow) and 93% (grey) identity (see also Table S4). *L. lactis* pND306 of *L. lactis* subsp. *lactis* 1252D (91) encoding the copper resistance associated *lcoRSABC* genes were almost identical to a similar locus (6.6 kb) encoded by pNZ712 (only 3 SNPs in the 6.6 kb *lcoRSABC* locus), of which also a part (*lcoRSA*) is present on pSK11P from *L. lactis* subsp. *cremoris* SK11 (7).

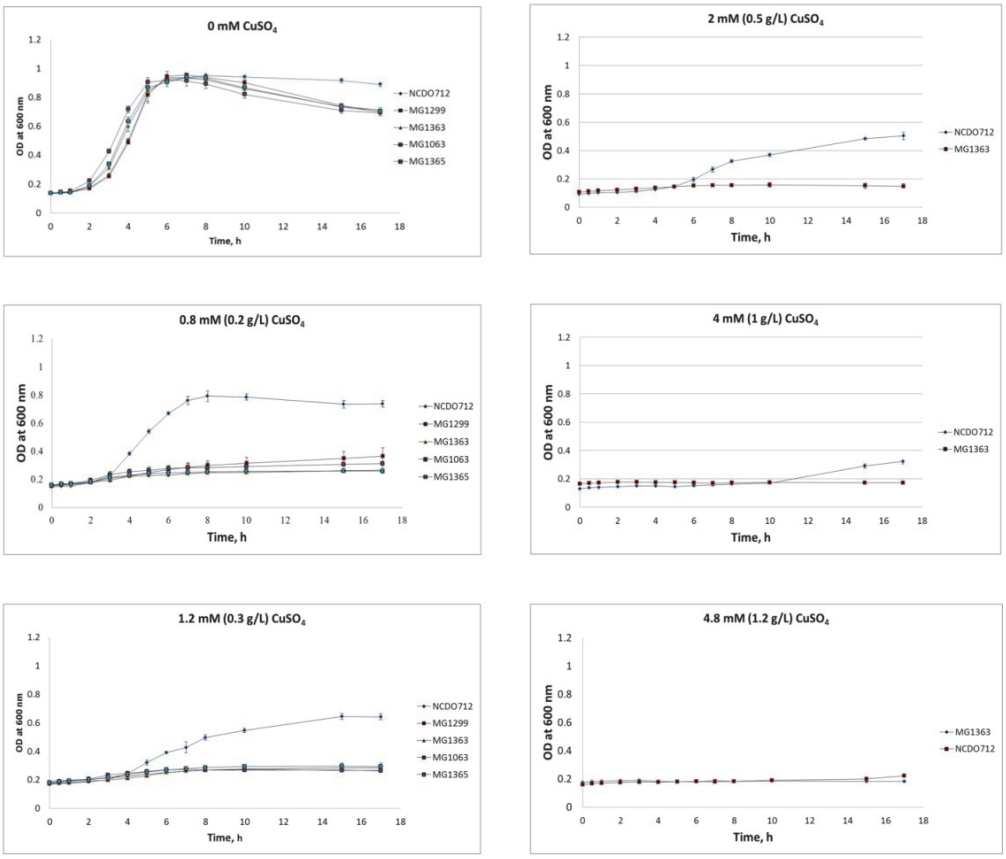

Figure S2. Growth of *L. lactis* NCDO712 and its derivatives carrying one or two plasmids (see Table 1) in medium with or without 0.8 (0.2 g/L) - 4.8 mM (1.2 g/L) $CuSO_4$. *L. lactis* NCDO712 is the only strain carrying pNZ712 with *lcoRSABC* coding for copper resistance genes. Each curve represents the average of 3 biological replications. Error bars show standard deviation.

Table S1. Oligonucleotides used in this study.

A) To identify *L. lactis* NCDO712 plasmids (34)	
pLP712_FW	GCTTTAATGGCTGCTCCATC
pLP712_RV	AGCACACCCGGATGATAGTC
pSH73_FW	TTTCAGTAGAAGGCCAAACAAC
pSH73_RV	TGCAAATTTATCTACAAAGGCTTG
pSH72_FW	GCTTTTTCGTTGGTTTGCTC
pSH72_RV	GCCCAAAATAGTGGGTTAGTG
pSH71_FW	TTGGGATAGAGCGTTTTTGG
pSH71_RV	CGGGGGAAATAAAATGACAAAC
pSH74_FW	GGACCAGATGGTACTTTTGAAGCG
pLSH74_RV	GGTAAAGTCACTATTGATGGACAGCC
pNZ712_FW	CACTCTAGTTTCCTACCTTCGTTGCAAGC
pNZ712_RV	CCAGTGCTAATCCTCCGTATAAGTATAGC
B) For pilin operon cloning	
pilin*PstI* forw*	CCGctgcagTTTGCAACAGAACCGTAATTGATTAGC
pilin*XhoI* rev*	CGGctcgagTTAAGTAATTTGAATTACTTGCTTTGAGAGTTGTTTAAAGG

*Indicated restriction enzyme sites are shown in small case in the oligonucleotide sequence.

Table S2. Single nucleotide polymorphisms between the chromosomes of *L. lactis* NCDO712 and its plasmids-free derivative MG1363 (66).

Predicted mutations					
Position[$]	**Mutation**	**Annotation[*]**	**Gene**	**Description**	**SNP type[#]**
26455	T→C	Intergenic (+111/-39)	*llmg_tRNA_07* → / → *mtlA*	tRNA-Asn/PTS system mannitol-specific transporter subunit IIBC	Intergenic, predicted to change promoter in -39 site; the -10 box is altered from an optimal TATAAT into TACAAT
522850	G→T	S214S (TCG→TCT)	*glpT* →	glycerol-3-phosphatase transporter	Synonymous
668056	A→C	D155E (GAT→GAG)	*tnp1297* ←	transposase for insertion sequence element IS1297	Non-synonymous; SIFT score - 1
671366	G→T	Q2K (CAA→AAA)	*llmg_0678* ←	transposase helper protein for IS981	Non-synonymous; SIFT score – 1
671428	T→C	intergenic (-59/-71)	*llmg_0678* ← / → *llmg_0679*	transposase helper protein for IS981/hypothetical protein	Intergenic
1167414	T→G	Intergenic (+64/-55)	*llmg_1198* → / → *llmg_1200*	hypothetical protein/hypothetical protein	Intergenic
1272351	C→T	L84L (TTG→TTA)	*llmg_1303* ←	hypothetical protein	Synonymous
1529872	A→C	I36M (ATT→ATG)	*llmg_1557* ←	hypothetical protein	Non-synonymous; SIFT score – 0.79
2143208	T→G	S244S (TCT→TCG)	*tnp904* →	transposase for insertion sequence element IS904I	Synonymous
2380149	C→T	G118D (GGC→GAC)	*tsf* ←	elongation factor Ts	Non-synonymous; predicted to affect protein function; SIFT score – 0.01
2492669	A→C	V284G (GTT→GGT)	*gapB* ←	glyceraldehyde 3-phosphate dehydrogenase	Non-synonymous; predicted to affect protein function; SIFT score - 0.02

[$] Indicates nucleotide position in the genome sequence of *L. lactis* MG1363 (RefSec: NC_009004.1)

[*] For mutations in intergenic regions the numbers indicates the relative position to the neighboring genes. For mutations in genes the amino acid (top) and nucleotide (bottom) changes are given.

[#] The amino acid substitution is predicted to be deleterious if the SIFT score is <= 0.05, and tolerated if the SIFT score is > 0.05. If intergenic mutations effect either the -10 or -35 box of the promoter it is indicated

Table S3. Main plasmid genes and features.

Plasmid	Genes	Product/Function
pLP712	*prtP*	Subtilisin-like serine protease
	prtM	Peptidyl-prolyl isomerase
	dld	D-lactate dehydrogenase
	lacR-lacABCDFEGX	Enzymes involved in lactose degradation
	pepF	Oligoendopeptidase F
	parAB	Plasmid partitioning proteins
	rep	Replication protein
pSH71[RS]	*repABC*	Replication proteins
pSH72	*repBXC*	Replication proteins
pSH73	*hsdS*	Type I R/M system specificity subunit
	repXB	Replication-associated protein RepX, RepB
	cadC, cadA	Resistance to heavy metals (cadmium), cadmium efflux ATPase (CadA)
pSH74	IS*1216*	1 IS element
	spaCB, spaA, srtC1, srtC2	Pilin gene cluster
	repB, repX	Replication proteins
	hsdS	Type I R/M system, specificity subunit
pNZ712	*nisCIP*	Nisin immunity
	lcoRSABC	Copper resistance
	mobD, mobC	Relaxase/mobilisation nuclease MobD and mobilization protein MobC
	repB, repX, repA	Replication proteins
	parA	Plasmid partitioning protein ParA
	mntH	Mn2+/Fe2+ transporter, NRAMP family
	five IS*S1*, three IS*981*, three IS*LL6*, two IS*1076*/IS*1069*/IS*1068*/IS*904*, IS*1251*, IS*712*, IS*1216*, IS*6770*	17 IS elements

Table S4. The main alignment results of pNZ712 and pSH74 to known lactococcal plasmids.

NCDO712 plasmid	Position	Alignment with plasmid	Position	Sequence identity in %/SNPs	Annotation
pNZ712	12054...21871; 1868...22735	pND306 (accession NG_035558.1) of *L. lactis* subsp. *lactis* 1252D (93)	1....9818; 9782....10650	99%, 3 SNPs, 1 gap	Copper resistance associated *lcoRSABC* genes
	12444...18177	pSK11P (accession NC_017500.1 and NC_008505.1) from *L. lactis* subsp. *cremoris* SK11 (8)	49965...55679	99%, 19 gaps and 8 SNP	*lcoRSA* genes
	46793...49826	pAH82 (accession AF243383)	7830...4798	99%, 3 gaps and 17 SNPs	CDS of unknown function
	25241...32960	pMRC01 from *L. lactis* DPC3147	45635...53355	99%, 3 gaps and 20 SNPs	Hypothetical and putative proteins
	44946...48234	pNP40 (accession DQ534432, AY530537)	61337...58037	93%, 50 gaps and 170 SNPs	*mobD*
pSH74	6395...6959	pKP1 (accession NC_016042.1)	8427...8991	99%, 6 SNPs	Transposase IS*1216*
	6398... 6959	pK214 (accession NC_009751.1) from *L. lactis* K214	29824...29263	99%, 6 SNPs	*tnpA*
	6395...6959	pSK11B (accession NC_013551)	9605...9044	99%, 6 SNPs	*tnp1-IS6*
	4793...5487	pCV56A (accession NC_017483.1) from *L. lactis* CV56	8618...9312	99%, 10 SNPs	Acetyltransferase
	6398...6959	pIBB-JZK (accession NC_024965.1) of *L. lactis* ILIBB-JZK	8369...7808	99%, 4 SNPs	Transposase IS*1216*
	5564...6310	pGdh442 (accession NC_009435) of *L. lactis* NCDO1867	33352...32607	98%, 13 SNPs, 3 gaps	Transposases *tnpR*
	5563...6275	pCIS5 (accession NC_019432) from *L. lactis* UC509.9	7678...8390	99%, 8 SNPS	Resolvase/integrase

Table S5. Microbial adhesion to HT29 and Caco-2 cells given in percent cells recovered after incubation with the cell lines. Standard deviation is given in parenthesis (n=3).

Strain	HT29 (% attachment)	Caco-2 (% attachment)
Lb. rhamnosus LGG	2.3±0.7	1.2±0.8
Lactococcus lactis NCDO712	0.3±0.2	2.4±2.2
Lactococcus lactis NCDO712(pIL253*pil*)	0.06±0.06	0.04±0.01
Lactococcus lactis MG1363(pIL253)	0.5±0.05	0.8±0.7
Lactococcus lactis MG1363(pIL253*pil*)	0.2±0.1	0.3±0.3
Lactococcus lactis MG1363	1.8±0.5	2.8±0.2
Lactococcus lactis MG1299	0.6±0.1	2.1±0.4
Lactococcus lactis MG1063	0.5±0.2	2.5±1
Lactococcus lactis MG1365	0.9±0.5	2.5±0.8

Table S6. Effect of the pilus gene cluster on conjugation of the lactose-fermenting ability to *L. lactis* MG1614.

Experiment #	Strain	Transconjugants per donor		Fold increased conjugation efficiency
		Wild type strain	Strain harbouring pIL253pil	
1	NCDO712	1.90E-07	2.50E-06	13.16
2	NCDO712	1.40E-07	6.00E-06	42.86
3	NCDO712	5.00E-08	4.60E-07	9.20
			Average fold increase	**21.74**

Experiment #	Strain	Transconjugants per donor		Fold increased conjugation efficiency
		Wild type strain	Strain harbouring pIL253pil	
1	MG1299	3.10E-07	1.60E-06	5.16
2	MG1299	1.50E-07	9.30E-07	6.20
3	MG1299	5.30E-08	1.90E-06	35.85
			Average fold increase	**15.74**

Chapter 3

Cell surface properties of *Lactococcus lactis* reveal milk protein binding specifically evolved in dairy isolates

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Abstract

Surface properties of bacteria are determined by the molecular composition of the cell wall and they are important for interactions of cells with their environment. Well known examples of bacterial interactions with surfaces are biofilm formation and the fermentation of solid materials like food and feed. *Lactococcus lactis* is broadly used for the fermentation of cheese and buttermilk and it is primarily isolated from either plant material or the dairy environment. In this study, we characterized surface hydrophobicity, charge, emulsification properties and the attachment to milk proteins of 55 *L. lactis* strains in stationary and exponential growth phases. The attachment to milk proteins was assessed through a newly developed flow cytometry-based protocol. Besides finding a high degree of biodiversity, phenotype-genotype matching allowed the identification of candidate genes involved in the modification of the cell surface. Overexpression and gene deletion analysis allowed to verify the predictions for three identified proteins that altered surface hydrophobicity and attachment of milk proteins. The data also showed that lactococci isolated from a dairy environment bind higher amounts of milk proteins when compared to plant isolates. It remains to be determined whether the alteration of surface properties also has potential to alter starter culture functionalities.

Introduction

The bacterial surface is important for interactions of the cell with the environment, especially when it comes to surface adhesion (1–3). Examples for such interactions are the fermentation of solid substrates like fermented foods (4), woody materials and straw (5), bioremediation of soil (6), the formation of biofilms (7–10) or during attachment of bacterial cells to the intestinal tract (11, 12). Microbial surface properties are especially important for the initial contact with and adhesion to a surface, which can occur via fimbriae, pili, flagella, or EPS (13). Once attached, cells can start to produce different polymeric components such as polysaccharides, glycoproteins, proteins, glycolipids, cellulose, and extracellular DNA (13), which can lead to biofilm formation and can further accelerate bacterial adhesion (7, 14).

The cell surface itself is characterized by properties like charge and hydrophobicity (15), which are determined by the molecular composition of the cell wall. The cell wall consists of peptidoglycan (16), polysaccharides (17), proteins, teichoic and lipoteichoic acids, lipids (18) and can be decorated with a sugar pellicle (19), pili (20–23) and/or an S-layer (24). The charge of the cell surface is determined by positively and negatively charged groups on teichoic and lipoteichoic acids, polysaccharides, proteins, and pili (19, 25, 26), while its hydrophobicity is related to the presence of polysaccharides, LPS, and (glyco-)proteinaceous material (18, 27), as well as pili (28) (Chapter 2). Although the bacterial surface contains positively and negatively charged molecules, the net surface charge of bacteria is mostly negative (29). The surface composition is species and strain specific (30), and can vary between different growth substrates (30) and growth phases (26, 31).

The interactions between cell surface and substrate can be electrostatic. For example, in sand, a strong negative charge of the cell surface causes electrostatic repulsion and thus prevents bacterial adhesion and increases cell transport through the sand matrix while cells with high hydrophobicity are retained by that matrix (32). Other types of interactions occurring are hydrophobic, van der Waals and Lewis acid–base forces. An example is the biofilm formation in which Brownian motion, hydrogen bonding and

electrostatic forces play a predominant role during the initial cell attachment, while cell hydrophobic forces as well as dipole-dipole, ionic bonding become more prominent during the phase of "irreversible" attachment of the bacteria to the surface (14, 29). In other words, during bacteria-substrate interactions, a combination of all forces is present: at initial interactions long-range forces are most important but once attachment is achieved, the short-range forces may predominate. Factors like pH, temperature and ionic strength influence the interactions and add complexity to explanations of bacteria-substrate interactions.

Lactococcus lactis is widely used as a starter culture in the production of cheese, sour cream and buttermilk (33), where it is responsible for food preservation, flavour formation and textural properties (33). It is classified into the subspecies (ssp.) *lactis* including ssp. *lactis* biovar. *diacetylactis*, ssp. *cremoris* and ssp. *hordniae*. The molecular composition of the *L. lactis* cell wall and its interactions with food components were reviewed by Burgain *et al.*, 2014. It was shown that within *L. lactis* the diversity in cell surface charge, hydrophobicity and the ability to stabilize emulsion is very high (2). Most *L. lactis* strains originate either from a dairy environment or from plant material, and literature suggests that strains of dairy origin have evolved from plant isolates (35). The transition from the plant to the dairy environment was analyzed by comparative genomics (36) or experimental evolution (37) and the results consistently describe similar metabolic adaptations. The main alterations during the plant-dairy transition are the loss of genes for the utilization of carbohydrate those only occur in plant material and the improved utilization of milk proteins. However, nothing is known about possible effects of the environmental transition on surface properties.

In this study, we investigated the surface properties of 55 *L. lactis* strains of which 25 were isolated from plant material and 30 from a dairy environment. We measured the cell surface hydrophobicity and charge as well as emulsion stabilizing properties and the attachment of the bacterial cells to milk proteins. Genotype-phenotype matching (GTM) (38–40) allowed identifying key molecules involved in *L. lactis* surface properties. An analysis based on phylogeny and strain origin revealed that dairy isolates have a much higher capacity to bind milk proteins.

Materials and Methods

Bacterial strains and culturing conditions

Lactococcus lactis used in this study (Table 1) were grown as standing cultures at 30°C in M17 (Oxoid, Thermo Scientific, Hampshire, UK) broth supplemented with 1% glucose (GM17) or 1% lactose (LM17). *E. coli* E10 containing pUC19 with an erythromycin resistance gene, pUC19E, was grown in tryptone yeast extract broth (TYB) at 37°C under vigorous shaking and access of oxygen. When required, antibiotics were added to the media: erythromycin (Em) was used at 10 μg/mL; chloramphenicol (Cm) - 10 μg/mL; nisin - 10 ng/mL. Optical density at 600 nm (OD_{600}) was measured using a single cell spectrophotometer (Ultrospec 2000, Pharmacia Biotech, Centerville, USA). Exponentially growing cells were prepared by diluting an overnight culture to an OD_{600} of 0.01 and subsequent incubation until an OD_{600} of 0.45±0.04 was reached. Stationary cells were prepared similarly by growing a culture for 16-18 h.

Table 1. The *55 L. lactis* strains and plasmid used in this study.

	Strain	Genotype	Origin	Reference
1	ATCC19435	*L. lactis* ssp. *lactis*	milk (dairy starter)	(41, 42)
2	HP	*L. lactis* ssp. *cremoris*	dairy starter	(41, 43)
3	P7266	*L. lactis* ssp. *lactis*	litter on pastures	(41, 42)
4	NCDO895	*L. lactis* ssp. *lactis*	dairy starter	(41, 42)
5	LMG8520	*L. lactis* ssp. *hordniae*	leaf hopper	(41, 42)
6	N41	*L. lactis* ssp. *cremoris*	soil and grass	(41, 43)
7	M20	*L. lactis* ssp. *lactis* biovar. *diacetylactis*	soil	(41, 42)
8	ML8	*L. lactis* ssp. *lactis*	dairy starter	(41, 42)
9	V4	*L. lactis* ssp. *cremoris*	raw sheep milk	(41, 43)
10	Li-1	*L. lactis* ssp. *lactis*	grass	(41, 42)
11	UC317	*L. lactis* ssp. *lactis*	dairy starter	(41, 42)

12	E34	*L. lactis* ssp. *lactis*	silage	(41, 42)
13	N42	*L. lactis* ssp. *lactis*	soil and grass	(41, 42)
14	DRA4	*L. lactis* ssp. *lactis* biovar. *diacetylactis*	dairy starter	(41, 42)
15	AM2	*L. lactis* ssp. *cremoris*	dairy starter	(41, 43)
16	P7304	*L. lactis* ssp. *lactis*	litter on pastures	(41, 42)
17	LMG8526	*L. lactis* ssp. *lactis*	Chinese radish seeds	(41, 42)
18	LMG9446	*L. lactis* ssp. *lactis*	frozen peas	(41, 42)
19	LMG9447	*L. lactis* ssp. *lactis*	frozen peas	(41, 42)
20	LMG14418	*L. lactis* ssp. *lactis*	bovine milk	(41, 42)
21	NIZO2244B	*L. lactis* ssp. *lactis*	mustard and cress	(41, 42)
22	FG2	*L. lactis* ssp. *cremoris*	dairy starter	(41, 43)
23	K231	*L. lactis* ssp. *lactis*	white kimchi	(41, 42)
24	KF7	*L. lactis* ssp. *lactis*	alfalfa sprouts	(41, 42)
25	KF24	*L. lactis* ssp. *lactis*	alfalfa sprouts	(41, 42)
26	KF146	*L. lactis* ssp. *lactis*	alfalfa and radish sprouts	(41, 42)
27	KW10	*L. lactis* ssp. *cremoris*	Kaanga way	(41, 43)
28	K337	*L. lactis* ssp. *lactis*	white kimchi	(41, 42)
29	KF67	*L. lactis* ssp. *lactis*	grapefruit juice	(41, 42)
30	KF134	*L. lactis* ssp. *lactis*	alfalfa and radish sprouts	(41, 42)
31	KF196	*L. lactis* ssp. *lactis*	Japanese kaiwere shoots	(41, 42)
32	KF201	*L. lactis* ssp. *lactis*	sliced mixed vegetables	(41, 42)
33	KF282	*L. lactis* ssp. *lactis*	mustard and cress	(41, 42)
34	LMG6897	*L. lactis* ssp. *cremoris*	cheese starter	(41, 43)
35	NCDO763	*L. lactis* ssp. *cremoris*	dairy starter	(41, 43)
36	SK11	*L. lactis* ssp. *cremoris*	dairy starter	(43, 44)
37	MG1363	*L. lactis* ssp. *cremoris*	cheese starter	(45)
38	KF147	*L. lactis* ssp. *lactis*	mung bean sprouts	(42, 46)
39	IL1403	*L. lactis* ssp. *lactis*	dairy starter	(47)
40	MG1299	*L. lactis* ssp. *cremoris*	dairy starter	(48)

41	B40	*L. lactis* ssp. *cremoris*	dairy starter	(43, 49)
42	NCDO712	*L. lactis* ssp. *cremoris*	dairy starter	(28)
43	SK110	*L. lactis* ssp. *cremoris*	dairy starter	(43, 50)
44	MG1362	*L. lactis* ssp. *cremoris*	dairy starter	(51)
45	MG1063	*L. lactis* ssp. *cremoris*	dairy starter	(51)
46	MG1261	*L. lactis* ssp. *cremoris*	dairy starter	(51)
47	MG1365	*L. lactis* ssp. *cremoris*	dairy starter	(51)
48	TIFN1	*L. lactis* ssp. *cremoris*	dairy starter	(52)
49	TIFN2	*L. lactis* ssp. *lactis* biovar. *diacetylacti*	dairy starter	(52)
50	TIFN3	*L. lactis* ssp. *cremoris*	dairy starter	(52)
51	TIFN4	*L. lactis* ssp. *lactis* biovar. *diacetylacti*	dairy starter	(52)
52	TIFN5	*L. lactis* ssp. *cremoris*	dairy starter	(52)
53	TIFN6	*L. lactis* ssp. *cremoris*	dairy starter	(52)
54	TIFN7	*L. lactis* ssp. *cremoris*	dairy starter	(52)
55	NZ9000	*L. lactis* ssp. *cremoris*	dairy starter	(53)
Plasmids used for gene overexpression				
	Plasmid	**Host organism**		**Reference**
	pNZ8150	*L. lactis*		(54)

Cell surface charge (mV)

Cells were harvested by centrifugation at 2,676 g for 3 min at room temperature and the cell pellet was washed 2x with 1 volume of 10 mM phosphate buffer (PB; pH=6.7) and re-suspended in the same buffer to an optical density OD_{600} of 1. Approximately 2 mL of this cell suspension was filled into the ZetaSizer DST1070 cuvette, which was inserted into ZetaSizer (Nano-ZS, Malvern, Malvern, UK). The electrophoretic mobility of cells was measured at 20°C and automatically re-calculated into the values of zeta potential (mV).

Cell surface hydrophobicity (CSH, %)

Cell surface hydrophobicity (CSH, %) was measured as described previously (55) with the following modifications: 5 mL of cell suspension (OD_{600} = 1) in PB was mixed with 2 mL of either petroleum or hexane (both from Sigma-Aldrich Chemie Gmbh, Munich, Germany) in surfactant-free glass tubes with a surfactant-free stopper. Tubes were vortexed for 2 min and kept still for 15 min at room temperature to allow phase separation to occur. Subsequently, 1 mL of the aqueous phase was transferred to a spectrophotometer cuvette and optical density (OD_{600}) of the cell suspension was measured at 600 nm. The surface hydrophobicity was calculated according to the following formula:

Cell Surface Hydrophobicity (CSH, %) = $(A_0-A_1)/A_0 \cdot 100$, in which A_0 represents the initial OD_{600} of cell suspension before mixing and A_1 is the OD_{600} of the water phase after mixing with petroleum or hexane and subsequent phase separation.

Emulsion stability (E24, %) was determined as described earlier (56, 57). Initially samples of cells in exponential and stationary growth phases were prepared in the same way as described for CSH with slight modifications. Briefly, 5 mL of cell suspension (OD_{600} = 1) in PB was mixed with 2 mL hydrocarbon, vortexed for 2 min and left standing for 24 h. The E24 index is given as a percentage according to: $E24(\%) = h_1/h_2 * 100$ %, in which h_1 is the height of the emulsified layer and h_2 is the total height of the emulsified layer and liquid column, both in mm.

Cell binding capacity to milk proteins

To one mL of cell suspension in either stationary or exponential growth phase prepared as described above (OD_{600}=1) 1 µL Syto 9 was added (Green Fluorescent Nucleic Acid Stain, Life Technologies, Bleiswijk, The Netherlands) after which the suspension was incubated in the dark for 30 min at room temperature. The cells were washed twice with PB to remove free dye.

Preparation of proteins: Sodium caseinate and sodium para-caseinate suspensions were prepared by dissolving 10 g of protein powder in 100 mL sterile demineralized water, followed by incubation for 20 min at 30-40°C to bring the proteins into solution and adjustment of the pH to 6.7 with 0.1 M NaOH or with 0.1 M HCl. One half of the prepared sodium caseinate solution was heated to 90°C for 10 min which allows denaturation of the residual whey proteins and their interaction with caseins. For protein staining 400 µL Nile blue A (Sigma-Aldrich Chemie Gmbh) was added to 100 mL of each protein solution, mixed, and incubated for 15 min at 21°C in the dark. To remove surplus dye the protein solution was transferred to a cellulose membrane tube with a molecular weight cut-off of 14 kDa (Sigma-Aldrich D9777-100FT, 25 mm width, 60 cm in length). Membrane tubes were pre-soaked in the sterile demineralized water for one hour at room temperature. The protein solution in the membrane tube was dialyzed against sterile PB for 24 h at 4°C in the dark. After dialysis, the protein concentration was quantified with Pierce BCA Protein Assay Kit (ThermoFisher, Bleiswijk, The Netherlands) according to the manufacturer's instructions. The Nile blue A-stained protein solution was divided over sterile Eppendorf tubes and stored at -40°C. The protein solutions were diluted to a final concentration of 1% prior to using them in the experiments.

For attachment measurements, 0.1 mL of Syto 9-stained cells and 0.1 mL of Nile blue A-stained proteins were mixed with a vortex for 10-15 sec and incubated for 1 min. Subsequently, this solution was analyzed in a Flow Cytometer (BD FACSaria II Cell Counter, BD BioSciences, Sparks, MD, USA). Excitation/emission wavelengths were 635/660±20 nm for Nile blue A and 485/530±30 nm for Syto 9, respectively. The results were analyzed using Flowing Software version 2.5.0 (http://www.flowingsoftware.com/).

Sorting procedure: Automatic (CST) and 'Accudrop Drop Delay' calibration (BD BioSciences, USA) of the flow cytometer was performed with 70 µm nozzle and threshold for FSC and SSC of 1,500. A total of 10,000 events from the area of interest were sorted in 1 mL of sterile PB. Serial dilutions (10^{-1}, 10^{-2}, 10^{-3}) of the sorted events were prepared in the sterile PB and 100 µL of each dilution was plated on LM17 or

GM17 agar plates. The agar plates were incubated overnight at 30°C and colony counts were determined.

Fluorescence microscopy

Cells and proteins were stained as described above; 100 µL of stained cells (OD_{600} = 1) were mixed with the same volume of 1% protein solution. Subsequently, 1 to 2 µL of this mixture was placed on a microscope slide, covered with a cover slip and examined at 100-fold magnification using an Olympus BX41 microscope (Olympus Corporation, Tokyo, Japan) with excitation wavelengths of 485±10 nm and 635±10 nm and emission wavelengths of 530±30 nm and 660±20 nm, respectively. Images were acquired with a charge-coupled-device camera with identical acquisition settings for all images; exposure to excitation light was for 200 ms for the Syto 9-stained cells and for 2,000 ms for the Nile Blue A-stained proteins. Image overlays were generated using ImageJ version 1.45s (https://imagej.nih.gov/ij).

Data analysis

Gene-trait matching (GTM) was performed using PhenoLink (40). Data visualization was done using R (https://cran.r-project.org/bin/windows/base/). The heatmap.plus function using Euclidian distance matrices and average hierarchical clustering and data scaling was used for the generation of heat maps.

Gene overexpression or deletion

Genes targeted for overexpression were PCR amplified using the hot-start KOD polymerase (Novagen, Madison, USA) according to the protocol of the manufacturer with primers listed in Table S1. Amplicons were purified using MSB® Spin PCRapase (Invitek, Gmbh, Berlin, Germany), digested with the restriction enzymes ScaI and XbaI (Fermentas GmbH, St. Leonn-Rot, Germany) and ligated into plasmid pNZ8150 (Table 1) digested with the same enzymes using T4 DNA ligase (Invitrogen, Breda, the Netherlands). DNA purification was carried out according to the protocols supplied by the manufacturers with Wizard®SV gel and PCR Clean-Up system (Promega, Leiden,

The Netherlands). Ligations were carried out at 16°C for 16-18 h and the ligated product, which was precipitated with 3 M sodium acetate (pH 5.5) and 70% ethanol, was used to transform electrocompetent cells of *L. lactis* NZ9000 (58). After electroporation, cells were plated on GM17 agar plates containing 10 µg/mL chloramphenicol and incubated at 30°C for 3 days. Single colonies were isolated and insert DNA in the plasmid was confirmed using colony PCR with the appropriate primers (Table S1, A).

Double crossover knock-outs of 4 genes were made in *L. lactis* MG1363 using pUC19 harbouring an erythromycin (Em) resistance gene, pUC19E. Upstream and downstream flanking regions (left flank, LF; right flank, RF) of the target genes were amplified with the primer pairs described in Table S1, B. Typically the left amplified flanking regions contained a sequence overlapping with the right flanking region (termed +vlag in the primer name in Table S1, B) which allowed to perform a splicing by overlap extension (SOE) PCR (59) to generate amplicons which were digested and subsequently ligated into similarly digested pUC19E. The ligation mixture was used to transform *L. lactis* MG1363 as described previously (58). Strains that were the result of a single crossover event were selected after plating and incubating the transformation mixture for 2 days at 30°C on GM17 agar plates supplemented with erythromycin. These single cross-over strains were grown for at least 100 generations in GM17 broth without Em to obtain strains that were the results of a double crossover event and that became Em-sensitive. The presence of the correct, clean gene deletions was confirmed by PCR using specific primers (Table S1, B).

Protein overexpression was verified with Sodium Dodecyl Sulfate - (10%) Polyacrylamide Gel Electrophoresis (SDS-PAGE) according to NuPAGE technical guide (Invitrogen, Carlsbad, CA) in cell extracts and supernatant fractions of exponentially growing cells (OD_{600} = 0.42 – 0.45) grown for 5h after addition of 10 ng/mL nisin.

Cell extracts were obtained by collecting the cell pellet from 10 mL culture (2,927 g for 5 min) and protein in supernatants was precipitated using trichloroacetic acid (TCA).

The cell pellet was re-suspended in sterile demineralized water to a final OD_{600} of 5 and transferred to microfuge tubes containing 1 g of zirconium beads and cooled on ice for 5 min. This was followed by 3x30 seconds of bead beating with a FastPrep FP100 bead-beater (Qbiogene, Cedex, France) and 1 min of cooling on ice in between the three cycles. After this treatment the tubes were left on ice for 5 min to allow the beads to sink and the supernatant was transferred to sterile Eppendorf tubes and kept at -20°C.

TCA precipitation was conducted by adding 2.5 mL 100% trichloracetic acid to 10 mL of culture supernatant, followed by vigorous mixing and incubation for 30 min on ice. Subsequently the tubes were centrifuged at 2,927 g at 4°C for 30 min, the supernatant was discarded carefully and the pellet was dissolved in 0.5 mL cold acetone. This was followed by centrifugation at the 2081 g at 4°C for 15 min, acetone was discarded; pellet was dried on air and re-suspended in sterile reverse osmosis water.

Results

Bacteria-protein interactions

As 30 out of 55 strains used in this study were isolated from a dairy environment, we decided to examine the affinity of *L. lactis* to different milk proteins. For this purpose, we developed a protocol that allowed quantifying the attachment of proteins to microbial cells. Bacterial cells were stained with the fluorescent DNA stain Syto 9, while proteins were colored with the fluorescent dye Nile Blue A. The emission spectra of these two dyes show little overlap and the individually stained particles could thus be distinguished by flow cytometry (Fig. 1, panel A). Cells give a high fluorescent emission signal at 530 nm while the proteins emit the highest signal at 680 nm. When Nile Blue A-stained protein binds to a Syto 9-labeled bacterial cell, the resulting particle should show high fluorescence at both wavelengths while this should not be the case when the two do not interact. This basic concept was tested using *L. lactis* TIFN5 (Fig. 1, panel B). We performed two additional experiments to validate the methodology with independent techniques. In the first experiment, Syto 9-labeled cells were mixed with stained proteins. The strain used was predicted to give limited to no binding to the

protein based on flow cytometry results. Using the flow cytometer we sorted 10 000 events from the population identified as protein based on the described method. The plating of these proteins showed that ≈5% of the sorted events led to the formation of a colony. On the other hand, sorting of events from the population identified as cells resulted in ≈95% of the cases leading to growth of a colony. These results were consistent with what was expected with an acceptable error margin of approximately 5%. In the second validation experiment, cells that were identified by flow cytometry as either binding or not binding to milk proteins were incubated with the protein of interest and subsequently visualized by light microscopy (Fig. 2). Clear differences in Nile Blue A fluorescence intensity indicate different levels of protein binding to the cells. The level of protein binding to a cell, as detected by fluorescence microscopy at 680 nm, (Fig. 2) is in agreement with the protein binding levels observed with flow cytometry, which decreases from strain HP to SK11, KW10 and P7266, respectively (Fig. S2, Table S2).

Sodium caseinate and sodium para-caseinate were selected as the proteins to be studied, as they represent the major milk proteins, i.e., the caseins. In sodium para-caseinate, the C-terminal caseinomacropeptide (CMP) of κ-casein is removed by enzymatic hydrolysis, as in cheese-making. Sodium caseinate was used in two forms: either or not pre-treated for 10 min at 90°C. The heat treatment was performed to account for possible heat-induced changes in the proteins, which is a common processing step when manufacturing fermented milk products (60, 61).

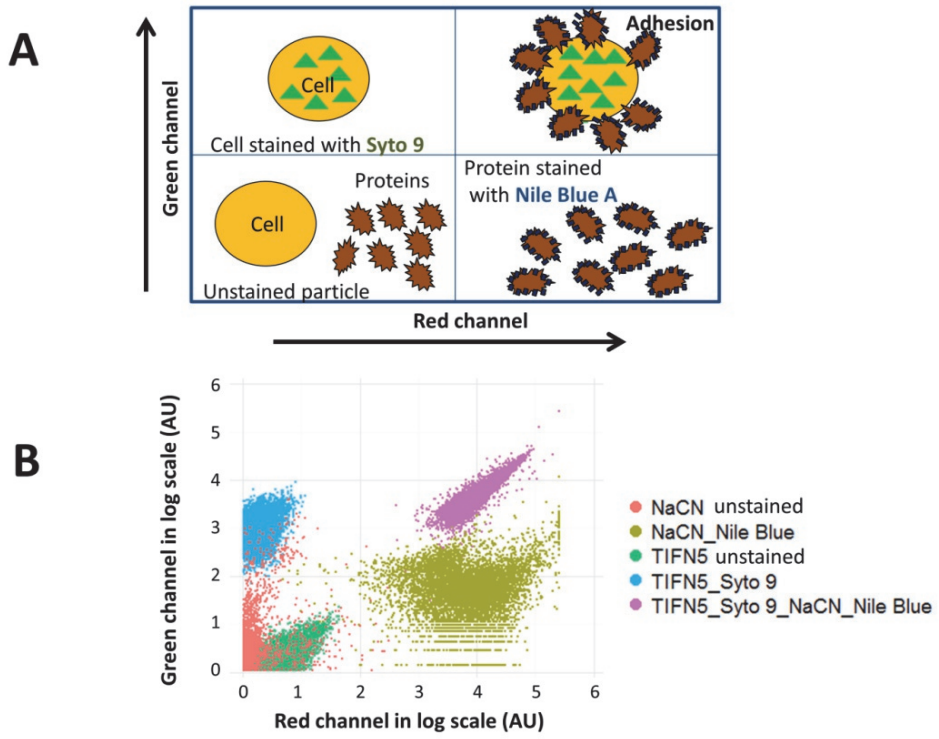

Figure 1. Measuring protein attachment to bacteria using flow cytometry. Panel A – Schematic view of bacteria-protein interaction and their expected appearance in a flow cytometer measurement. Unstained proteins and cells are expected in the lower left quadrant. The lower right quadrant shows proteins stained with a red fluorescent dye while the upper the left quadrant shows cells stained with a green fluorescent dye. Bacteria covered with surface-bound protein should appear in the upper right quadrant while two separate clouds should be seen if the proteins do not attach to the cell surface. Panel B – Example of attachment of sodium caseinate (NaCN) to *L. lactis* TIFN5. Values on the both axes are log-transformed. Unstained proteins (red) and cells (dark green) are located in the bottom left quadrant; stained cells are shown in blue, stained protein are colored light green, and events representing proteins-attached-to-cells are located in the upper right corner (purple).

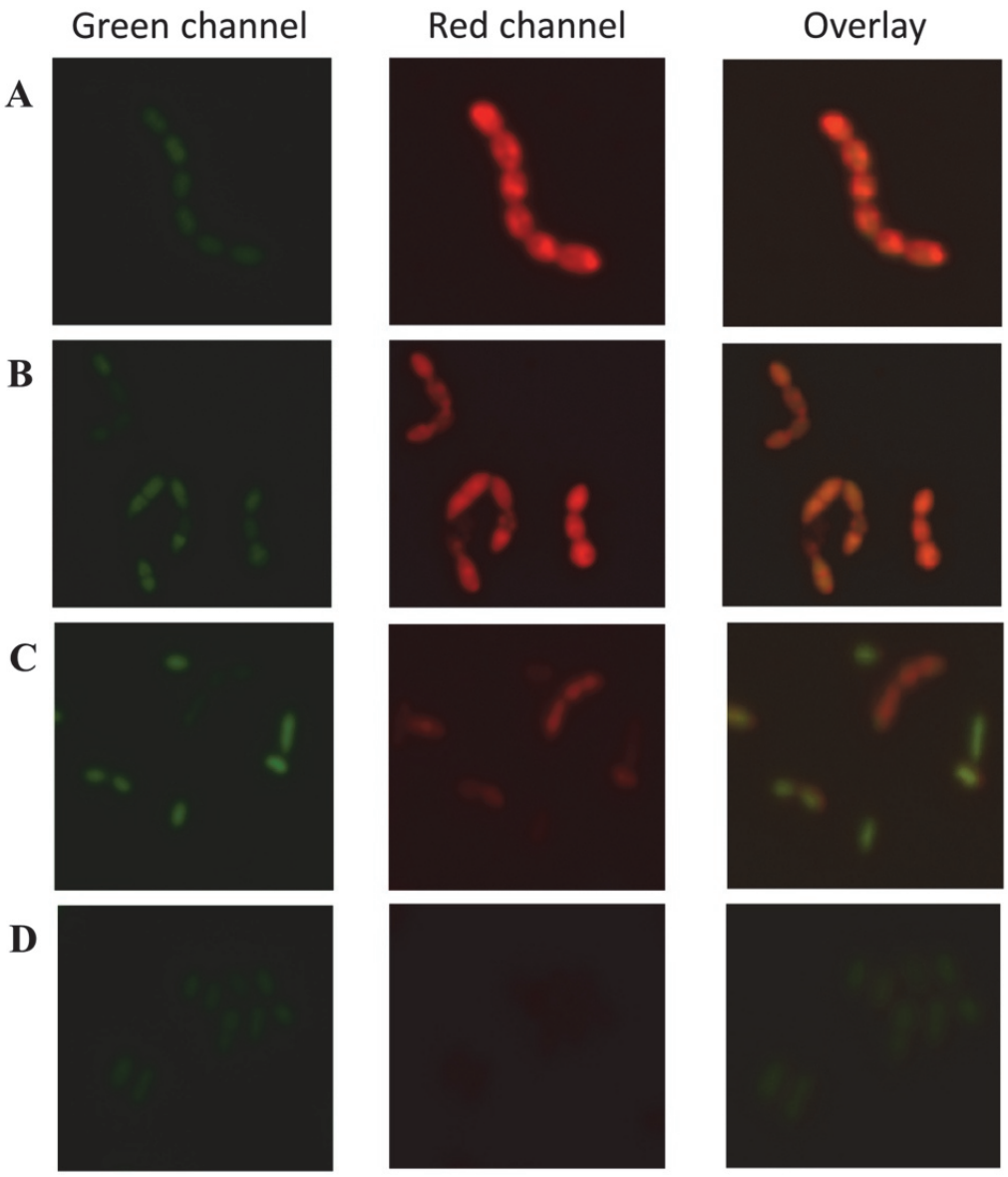

Figure 2. Fluorescence microscopy to determine protein (sodium caseinate) attachment to *L. lactis*. Strains are sorted from A-D by decreasing attachment strength as determined by the flow cytometry-based method. Panel A – *L. lactis* HP, panel B – *L. lactis* SK11, panel C - *L. lactis* KW10, Panel D – *L. lactis* P7266. The green channel represents the DNA stain, the red channel - the stained protein bound to the cell surface, and the overlay combines both signals. The results indicate decreased attachment of sodium caseinate to the strains from panels A-D respectively, which is consistent with the findings of the flow cytometric approach.

Using this approach, 55 strains of *L. lactis* were screened for their ability to bind the three milk proteins. The strains bound all proteins to some extent, but clear differences were observed (Table S2, Fig. 3, Fig. S1 – S3). Significant differences in growth phase-dependent protein binding were seen for 12 of the 55 strains (Fig. S4). While the ability of *L. lactis* to bind proteins is strain-specific the results also show that the capacity of binding milk proteins appeared to be larger than 61% with the average between 82-97% for strains of dairy origin. These mostly belong to the *L. lactis* ssp. *cremoris* and ssp. *lactis* biovar. *diacetylactis.* The 10 out of 23 *L. lactis* strains of plant origin, which belong to the ssp. *lactis* (indicated as dark and light green in the "origin" and "species" column in Fig. 3, respectively), showed either poor (less than 50 %) or no protein binding for both growth phases (Fig. 3, Fig. S1 – S3, Table S2).

Cell surface hydrophobicity, emulsification properties and surface charge

Cell surface charge, hydrophobicity, and emulsion stabilizing ability of the individual strains were determined on exponentially growing and stationary cells (Table S2). The results show a considerable phenotypic diversity (Fig. 3, Fig. S1 – S3, Fig. S5 – S7). The cell surface charge – measured as zeta potential – varied from -3.4 mV to -42.3 mV between the different strains. Cell surface hydrophobicity (CSH) is the measure of the extent to which cells suspended in a water phase are attracted to a hydrocarbon phase when both phases are mixed vigorously and left for phase separation to occur. We found that 25 out of the 55 strains have a cell surface hydrophobicity ranging from 0% to 20% for both growth phases. On the contrary, 9 strains showed >95% hydrophobicity for stationary growth phase, while another 5 strains showed such a high hydrophobicity in the exponential growth phase. In stationary growth phase strains NCDO712 and MG1299 displayed 60-99% hydrophobicity while the plasmid cured derivatives MG1063, MG1261, MG1362. MG1363, MG1365, and NZ9000 showed a hydrophobicity of 5-35% (Fig. S5, Fig. S8, Table S2). This indicates that hydrophobicity in NCDO712 might be a plasmid-encoded trait.

Hierarchical cluster analysis revealed no correlations between the CSH of the strains as determined with different hydrocarbons and their binding of proteins (Fig. 3). Strains in

clade 1 show low surface hydrophobicity in the exponential growth phase, but high binding affinity to milk protein is seen. This clade consists mainly of *L. lactis* ssp. *cremoris* strains of dairy origin. In contrast, strains in clade 2 poorly bind to milk proteins while they have a high cell surface hydrophobicity. Clade 3-strains show poor protein binding capacity and a low surface hydrophobicity. Clade 2 and 3 consist mainly of *L. lactis* ssp. *lactis* strains originating from the plant environment. For cells from stationary growth phase some differences are seen but the overall trends are the same in the clades 1, 3 and 4 (Fig. 3, panel B). Together, the results demonstrate that surface hydrophobicity of *L. lactis* cells and their protein binding capacity are independent parameters. Interestingly, the analysis of the origin of the strain (plant or dairy) and species of *L. lactis* revealed that the majority of *L. lactis* ssp. *cremoris* and *L. lactis* ssp. *lactis* biovar. *diacetylactis*, both of dairy origin, have a high capacity to bind to milk proteins. In contrast, the majority of strains originating from plan material only poorly bind milk proteins (Fig. 3).

The stability of the emulsions obtained by mixing the water and hydrocarbon phases varied between the strains from no observed emulsification to total hydrocarbon phase emulsification and stability for at least 24 hours. No correlation was found between emulsion stabilizing capacity of the strains and their cell hydrophobicity, between their charge and hydrophobicity, and between their charge/hydrophobicity and capacity to bind proteins.

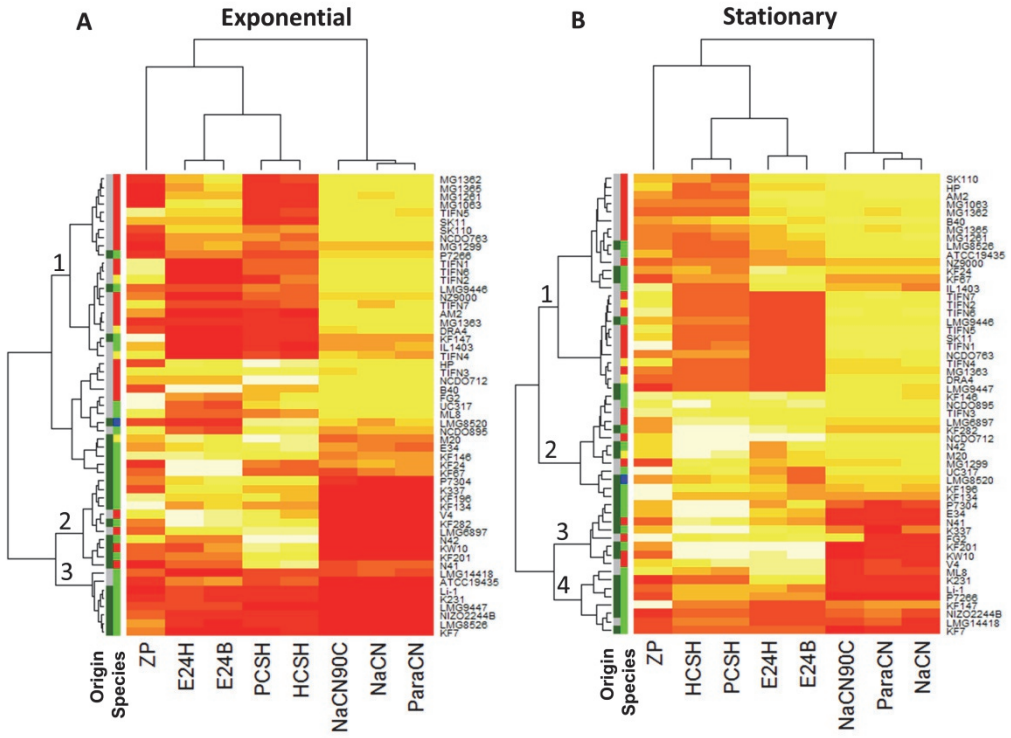

Figure 3. Heat map of surface properties of *55 Lactococcus lactis* strains: Cell charge (ZP), hydrophobicity measured with petroleum (PCSH) or hexane (HSCH), emulsion stability measured after 24 h (E24B –measured with Petroleum or E24H – measured with Hexane), attachment to milk proteins: para-caseinate (ParaCN), sodium caseinate (NaCN) and sodium caseinate heated for 10 min at 90°C (NaCN90C). Panel A comprises results using the cells from the exponential growth phase, while for Panel B cells from the stationary growth phase were taken. Low values are represented by a darker/red color while higher values are represented by a lighter/yellow color of the heat map segment (n=3). For the charge (ZP) a darker color represents more negatively charged cells. The Origin/Species columns indicate, respectively, plant (green) or dairy (gray) origin and the species *lactis* (green), *cremoris* (red), *hordniae* (blue) or *lactis* biovar. *diacetylactis* (yellow).

A comparison of stationary-phase and exponential-phase cells revealed that 12 of the 55 strains show significant growth phase-related differences in their capacity to bind the milk protein samples tested, 3 strains show significant changes in hydrophobicity and 9 of the 55 strains show differences in the E24 measured (Fig. S4, Fig. S8). The charge of stationary and exponentially growing cells differed significantly for 22 out of 55 strains tested (Fig. S7). Taken together, the results indicate that the measured cell surface properties have some growth phase dependency, but strain specific properties are much more determinant. The binding of different milk proteins (regression coefficient r = 0.88-0.93) and cell hydrophobicity measured with different hydrocarbons (r = 0.98) are in relatively good agreement. However, little to no correlation is observed between hydrophobicity, cell surface charge and protein binding. Interestingly, there is a clear overrepresentation of strains of dairy origin in the clusters that show high binding ability of milk proteins which suggests that this trait might be beneficial during evolution in a dairy environment.

Genotype-Phenotype matching

A random forest-based genotype-phenotype matching algorithm (40) was employed to identify genes potentially involved in cell surface properties. The analysis was run separately for each individual phenotype measured, resulting in candidate lists for genes involved in the individual traits. This resulted initially in 201 candidate genes which were selected based on the highest importance score and gene description. From these 201 candidate genes, 18 were selected for further characterization on the basis of three parameters: i) the importance score in the individual GTM analyses; ii) multiple appearances in the GTM analysis for the individual phenotypes; and iii) the predicted gene function being related to cell surface (Table S3). The following choices were made for the further characterization of these genes. If the presence of a selected candidate gene was found to be associated with a phenotype and this gene is absent in *L. lactis* MG1363, it was overexpressed in MG1363. Conversely, when gene absence was found to be associated with a phenotype and this gene is present in MG1363, it was knocked-out in MG1363. We selected the longest gene within the orthologous group of the 55

strains for overexpression purposes, to eliminate the risk of working with a truncated protein. Eight of the 18 genes could not be deleted and/or overexpressed, possibly because they are essential or deleterious. Ultimately, 6 of the selected candidate genes were successfully cloned downstream of the nisin-inducible promoter P_{nisA} in pNZ8150 (Table 1) in *L. lactis* NZ9000, the *L. lactis* MG1363 derivative, while 4 of the genes were deleted from the chromosome of *L. lactis* MG1363 (Table 2). While we cannot exclude that the addition of nisin itself has an effect on surface properties we would like to point out that the overexpression results reported here are in relation to the nisin induced control strain which carried an empty plasmid vector. In addition, no significant differences in surface properties are seen between the uninduced *L. lactis* MG1363 and its nisin induced derivative NZ9000 harbouring pNZ8150 (Table S2, Table S4, Table 1). Overexpression of proteins of the predicted sizes, and their cellular localization were examined with SDS-PAGE after induction with 10 ng/mL nisin of exponentially growing cells carrying the expression plasmids and incubation for another 5 h (Fig. S9). The results showed that all 6 proteins could be successfully overexpressed, as they were detected either intracellularly or in the medium.

Table 2. Genes that were overexpressed or deleted from the chromosome.

Gene name	Present in strain	Locus tag Protein ID	Protein size (aa/kDa)	Growth phase [¥]	Gene presence (Pr) Gene absence (Ab) [¥]	Predicted phenotype [¥,$]	Detected phenotype [*,$]	Detected in growth phase [*]
Overexpression in *L. lactis* NZ9000								
Cell surface protein precursor	B40	B40_0084 LITC01000011 KZK48299.1	930/ 102.17	ST	Pr	ZP▼	CSH▲	ST
Ribose 5-phosphate isomerase A (*rpiA*)	KF147	KF147_0667 ABX75739.2	243/ 26.95	EX	Ab	ParaCN▲	CSH▲	ST
Internalin, putative (LPXTG motif)	KF282	KF282_0409	559/ 61.28	ST	Pr	ZP▲	CSH▲	EX, ST
Hypothetical protein (*yreB*)	IL1403	L128699 AAK05785.1	314/ 35.64	EX	Pr	CSH▲	None	-
Cell wall surface anchor family protein	KF147	LLKF_0311 ADA64081	809/ 87.46	EX EX	Pr Ab	CSH▲ E24▲	CSH▲ ParaCN▼ NaCN▼ NaCN90C▼	EX, ST ST ST ST
Endo-beta-N-acetylglucosaminidase (*ypcCD*)	KF147	LLKF_1605 ADA65249	923/ 102.53	ST ST	Ab Ab	ParaCN▲ NaCN▲	CSH▲	ST
Knock-out in *L. lactis* MG1363								
Conjugal transfer protein (*traG*)	MG1363	llmg_1383 CAL97970	612	EX	Ab	ParaCN▼	ParaCN▼ NaCN▼ NaCN90C▼	EX EX EX
Cell surface protein precursor	MG1363	llmg_1096 CAL97690	387	ST	Ab	ZP▼	No	-
Hypothetical protein	MG1363	llmg_1093 CAL97687.1	334	ST	Pr	NaCN▲	NaCN▼	EX, ST
Sortase SrtA (*srtA*)	MG1363	llmg_1449	250	-	-	-	No	-

[¥] These columns indicate if either the presence or absence of a particular gene of cells from exponential (EX) or stationary (ST) growth phase resulted in an altered phenotype

[*] These columns indicate phenotypic changes that were detected in a particular growth phase in engineered strains where the indicted genes were either overexpressed or deleted

[$] ZP – charge (mV), ParaCN – attachment to paracaseinate (%), NaCN – attachment to sodium caseinate (%), NaCN90C – attachment to sodium caseinate heated at 90°C for 10 min (%), CSH – cell surface hydrophobicity (%), E24 – emulsion stability for 24h (%), ▲ – cell surface property increases, ▼ – cell surface property decreases. All phenotype changes indicated are significant with $p<0.01$

Cell surface properties of the recombinant *L. lactis* strains

The deletion of the gene *llmg_1383* decreased the binding of exponentially growing cells of MG1363 to the 3 milk proteins tested by 53±3 %. The protein Llmg_1383 of *L. lactis* MG1363 is annotated as a conjugal transfer protein TraG, which is described to aid in the transfer of DNA during bacterial conjugation. As it is predicted to be involved in membrane pore formation (type IV secretion) (62), a role in surface alteration seems plausible.

Similar to *llmg_1383*, the deletion of the hypothetical gene *llmg_1093* also decreased the binding of *L. lactis* MG1363 to the three milk proteins examined by 60±27% for cells in the exponential growth phase and by 42±37% for cells in the stationary growth phase. Protein Llmg_1093 is a putative secreted protein but its actual function is not known.

As several of the genes studied here harbor sortase dependent LPXTG signals we also deleted the sortase A gene (*llmg_1449)* from the chromosome of strain *L. lactis* MG1363. This deletion did not change the cell surface properties of *L. lactis* MG1363 (Table S4), indicating that SrtA might not be involved directly in cell surface properties. We also tried to obtain a knock-out mutant of the *srtC* gene but were not able to obtain it after two attempts, indicating a possibly essential role of SrtC for growth.

The cell surface protein precursor B40_0084 is a putative mucus binding protein as it has 4 mucus binding domains (MucBP) (pfam06458) and a LPXTG-motif cell wall anchor domain (TIGR01167). GTM predicted that the presence of this protein leads to a more negative cell surface charge. This prediction was not confirmed, but changes were observed for hydrophobicity. For example, for stationary phase cells where the control strain shows a CSH of 8.8% (±5%), the overexpression of B40_0084 resulted in a CSH of 89% (±7%) (Table S4). Interestingly, while SDS-PAGE analysis (Fig. S9) verified the overexpression of B40_0084 in exponentially growing cells, this overexpression did not affect the CSH of the cells in this growth phase. Mucus binding proteins are described to be involved in the binding of carbohydrates such as mannose

(63) and they are speculated to be important for probiotic function (64). The effect of the overexpression of *B40_0084* only leads to hydrophobicity changes in stationary phase cells, indicating that other surface decoration(s) dominates surface properties in a growth phase-dependent manner.

Another gene identified by gene-trait matching is a putative internalin containing four MucBP domains (pfam06458) and a surface-anchoring domain (COG4932). Internalins were originally described in *Listeria monocytogenes* as surface proteins that are involved in adhesion to mammalian epithelial cells (65). For example, protein internalin A (InlA) mediates bacterial adhesion and invasion of epithelial cells in the human intestine through specific interaction with its host cell receptor E-cadherin (66). Here, we overexpressed the ortholog from strain KF282 (KF282_0409). While gene-trait matching associated the presence this gene with lower surface charge, its overexpression in MG1363 increased the CSH, from 8.8% (±5%) in the control strain to 49.3% (±21.8%). Furthermore, overexpression of a cell wall surface anchor family protein (LLKF_0311) led to the decreased attachment of cells to milk proteins from 98% (±0.7%) to about 31% (±47%) in the stationary growth phase and to the increase in cell surface hydrophobicity from 9±5% to 78±9% in the stationary and exponential growth phases (Table S4) while initially the presence of *LLKF_0311* was predicted to effect cells surface hydrophobicity and emulsion stability in exponential phase of growth. (Table S4).

The endo-beta-N-acetylglucosaminidase *ypcCD* (*LLKF_1605)* was found to be associated to the binding of cells to milk proteins. Overexpression of this protein did not lead to an alteration in attachment of cells in stationary growth phase to milk proteins, but led to an increase in surface hydrophobicity from about 8.8±5% to 23.7±16.1%.

Overall, the phenotype predicted by gene-trait matching could be confirmed experimentally for 3 out of 10 engineered strains, and for 2 out of 3 strains additional altered surface properties were detected. Overexpression of the selected candidate genes did not influence the protein binding properties. A total of 4 strains showed

altered surface properties but not the predicted ones whereas for 3 strains no changes were observed. While not all predictions were correct, the approach did allow identifying targets that are of importance for lactococcal surface properties.

Discussion

This study describes bacterial surface properties such as cell surface charge, hydrophobicity and the attachment to milk proteins for 55 *L. lactis* strains isolated from either plant material or the dairy environment. A flow cytometry based method for the characterization of protein binding allowed to demonstrate the existence of a large biodiversity in cell attachment to milk proteins. We show that the capacity of cells to bind the milk proteins is growth phase-dependent for some of the strains tested. Importantly, this methodology is not restricted to the use of milk proteins and we expect it to be applicable for the characterization of cell attachment to other proteins, and for other bacterial species. In combination with cell sorting the method may prove useful in enabling the selection of cells with desired surface characteristics.

In contrast to a previous executed GTM study with *L. lactis* strains, which was done based on comparative genome hybridization data (40), we were able to use either draft or complete genome sequences, which should increase the predictive power of the approach. The characterization of selected target genes, by their overexpression and/or deletion from the chromosome, resulted in the identification of 7 genes that are involved in cell surface properties.

The observed biodiversity of cells obtained from different growth phases might be explained by differences in the molecular composition of the cell wall. For example, peptidoglycan modification during exponential growth in *L. lactis* results only in partial (75%) amidation of the alpha-carboxyl group of the D-Asp cross-bridge to the PG precursor (67, 68). In contrast, the amidation of amino acids during peptidoglycan modification for *L. casei* is almost complete (near 100%) during both growth phases (16). However, peptidoglycan is not the major component exposed at the bacterial surface, but it is rather dominated by polysaccharides, teichoic acids and proteins. The

charge of the cell surface is mainly determined by net charge of molecular composition of cell wall. For example, a negative cell surface charge can be partially determined by carboxyl and phosphate groups of LTA and TA, while positive charges partially derive from D-alanine molecules that are esterified to TA and LTA (25).

Overall we see poor or no correlations between properties such as emulsion stabilizing ability and cell hydrophobicity, between charge and hydrophobicity, or between charge/hydrophobicity and the attachment to proteins. This might be caused by the amphiphilic surface properties of bacteria (69).

While there is ample literature describing the cell surface and cell wall composition of bacteria in general, available information on key molecules determining cell surface properties of lactic acid bacteria is limited. For instance, the overexpression of the surface anchored protein CwaA from *Lactobacillus plantarum* NL42 in *L. lactis* NZ9000 led to cell auto-aggregation, increased hydrophobicity and attachment of the CwaA-producing *L. lactis* cells to human epithelial HT-29 cells (70). Other studies describe the autolysins AcmA and AcmD, which are involved in cell chaining (71), or the expression of pili on the surface of *L. lactis*. Pili can be plasmid- as well as genome-encoded and they have been shown to cause auto-aggregation (21, 28) (see also Chapter 2) and to increase attachment to epithelial cells (22, 70). The *L. lactis* cell wall proteinase PrtP was also shown to be involved in cell surface properties and adhesion to solid surfaces (72).

We successfully verified the influence of 3 proteins on predictions based on the performed gene-trait matching. However, the alteration of protein expression in some of our engineered strains did result in an effect on the cell surface other than the predicted one. The underlying molecular details of these discrepancies are not clear, but we speculate that affected molecules are in competition for space on the cell surface. Alteration of the expression level of one molecule would indirectly affect the overall surface composition, which could result in unexpected phenotypic outcomes. Such a speculation is in line with recent theory on trade-offs that can be determined by physical-chemical constraints such as membrane space (73, 74). While most of the

genes identified here could be linked to the cell surface we could not find such a link for some identified proteins based on sequence analysis. The overexpression of a ribose-5-phosphate isomerase, an enzyme involved in the pentose phosphate pathway, altered cell surface hydrophobicity. A direct role of this enzyme in cell surface properties seems unlikely, but a study in *L. plantarum* suggests that ribose acts as a precursor for alternative cell wall teichoic acids (12) and it is therefore conceivable that a change in ribose availability could lead to altered cell wall properties. The identification of such proteins, that cannot be linked to cell surface properties with e.g. sequence based motif analysis, points out the added predictive value of genotype-phenotype matching.

An interesting observation is the fact that strains isolated from a dairy environment show much stronger binding of milk proteins as compared to plant isolates. Literature holds many examples for the role of surface alterations to improve the fitness of an organism in a particular environment. In pathogenic bacteria, for instance, peptidoglycan modifications allow escaping the host's immune system (75) and in soils the capacity to form biofilms is a key factor for microbial fitness (76). The proposed evolutionary transition of *L. lactis* from the plant to the dairy environment is described to be accompanied by the loss of the ability to synthesize several amino acids or to catabolize typical plant-derived sugars. The occurrence of amino acid auxotrophies in dairy isolates is compensated by improved utilization of milk proteins through e.g. extracellular proteases, dedicated (oligo)peptide transport systems and intracellular peptidases (35, 37). The fact that binding to milk proteins was selected for in a dairy environment suggests a selective advantage, which seems plausible seen the growth dependency of dairy strains of *L. lactis* on extracellular amino acids. It will be interesting to see if the alteration of surface properties of *L. lactis* also impacts on the functionality of starter cultures in pure and mixed-culture fermentations.

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Conflict of interest

The authors declare no conflict of interest.

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Supplementary information for Chapter 3

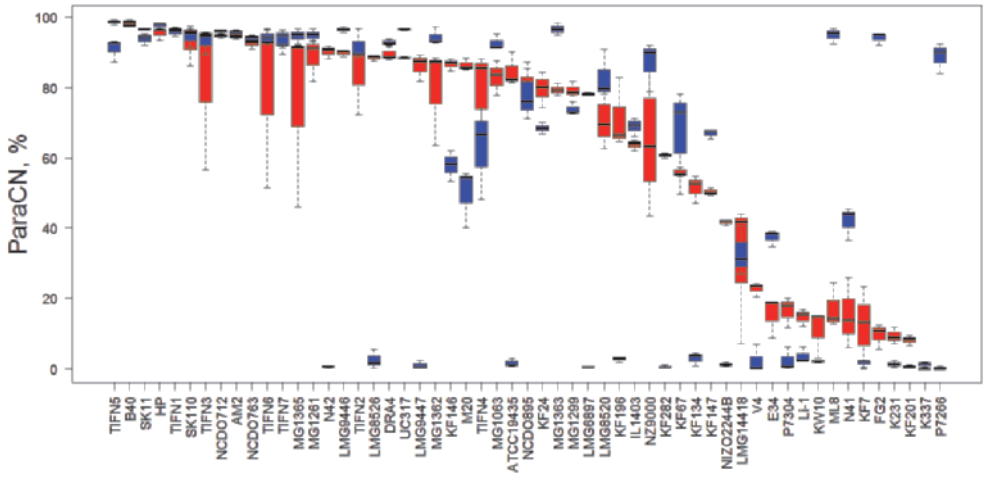

Figure S1. Attachment of para-caseinate (ParaCN, %) (y-axis) to *55 Lactococcus lactis* strains (x-axis) (n=3). Bacterial strains were sorted from the highest to the lowest value for cells from stationary growth phase (shown in red). The values measured for cells from exponential growth phase are shown in blue.

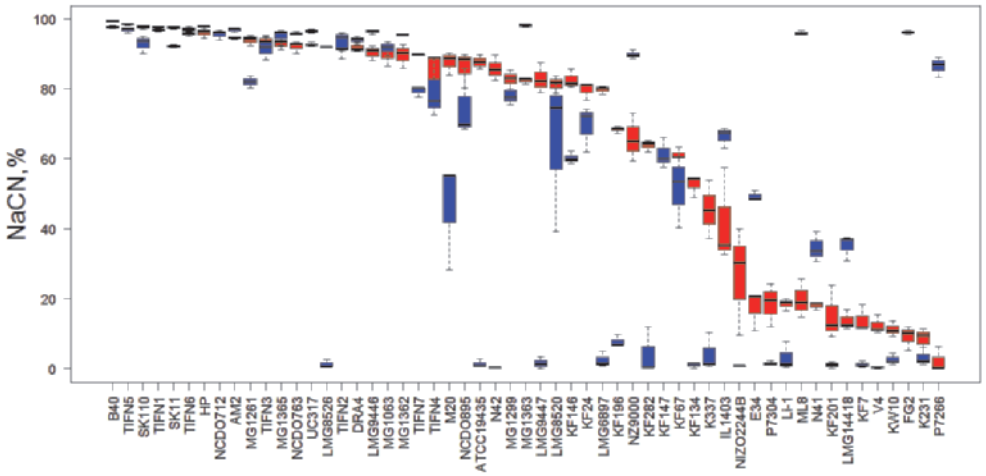

Figure S2. Attachment of sodium caseinate (NaCN, %) (y-axis) to *55 Lactococcus lactis* strains (x-axis) (n=3). Bacterial strains were sorted from the highest to the lowest value for cells from stationary growth phase (shown in red). The values measured for cells from exponential growth phase are shown in blue.

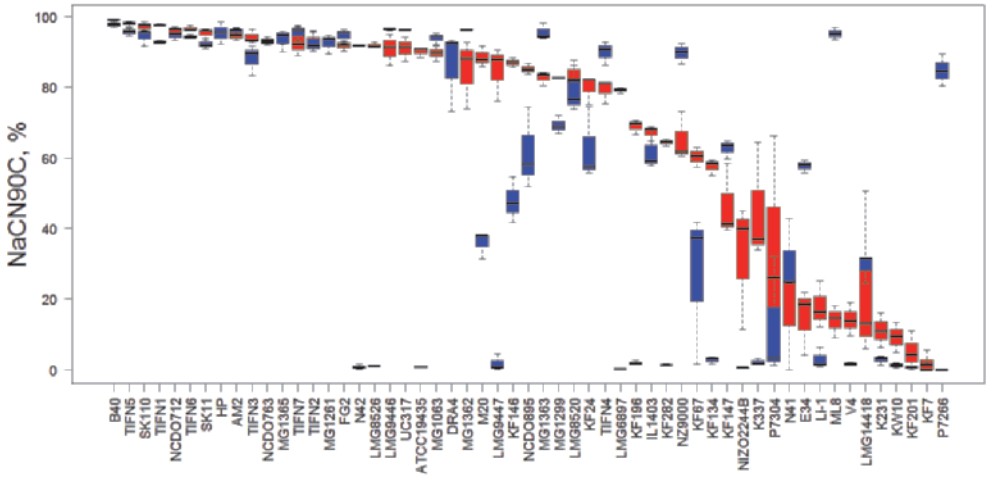

Figure S3. Attachment of sodium caseinate heated for 10 min at 90°C (NaCN90C, %) (y-axis) to *55 Lactococcus lactis* strains (x-axis) (n=3). Bacterial strains were sorted from the highest to the lowest value for cells from stationary growth phase (shown in red). The values measured for cells from exponential growth phase are shown in blue.

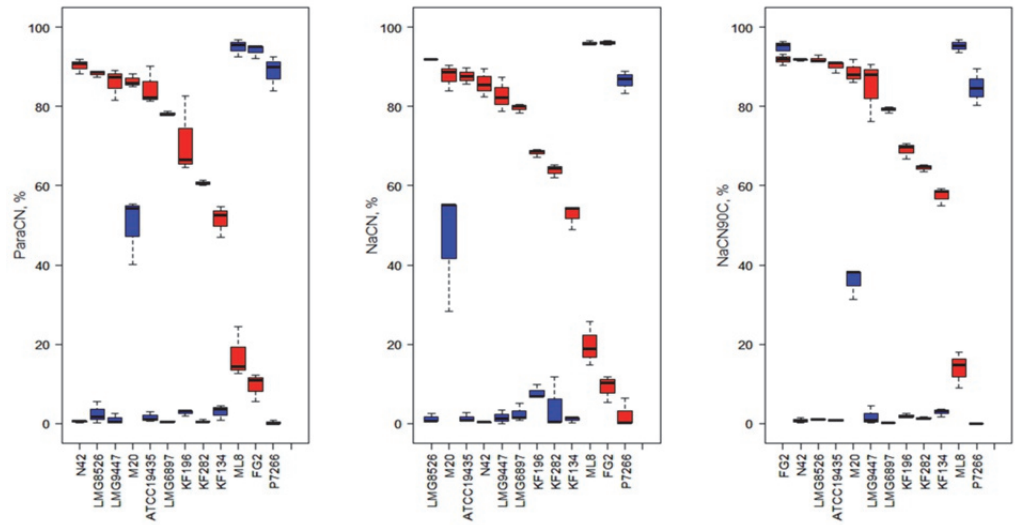

Figure S4. Twelve out of *55 Lactococcus lactis* strains (x-axis) which showed opposite attachment behavior to milk proteins (y-axis) when either originating from exponential or stationary growth phases, (n=3): ParaCN,% indicates attachment to para-caseinate, NaCN,% - attachment to sodium caseinate, and NaCN90C,% - attachment to sodium caseinate heated for 10 min at 90°C. Bacterial strains were sorted from the highest to the lowest value for stationary growth phase (shown in red). The values measured at exponential growth phase are shown in blue.

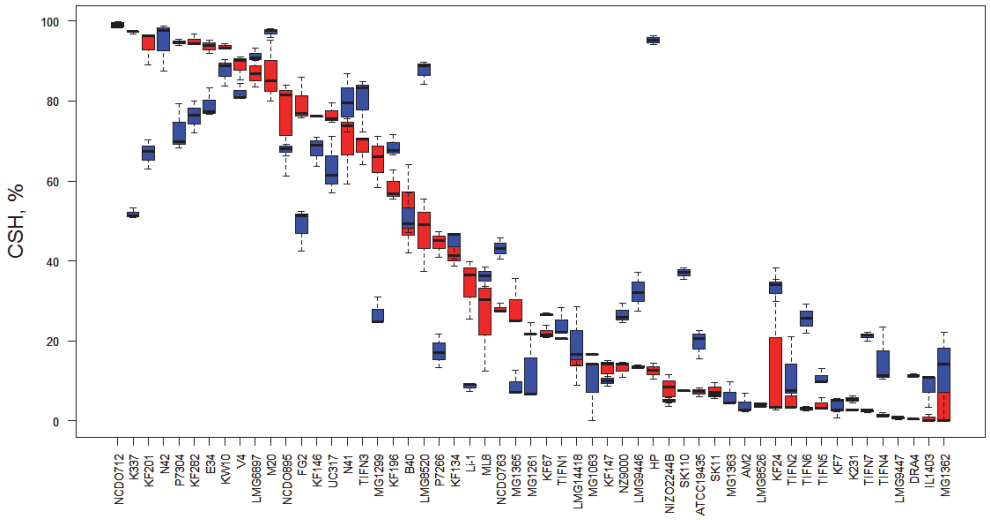

Figure S5. Hydrophobicity (CSH, %) (y-axis) of *55 Lactococcus lactis* strains (x-axis) (n=3). Bacterial strains were sorted from the highest to the lowest value for cells from stationary growth phase (shown in red). The values measured for cells from exponential growth phase are shown in blue.

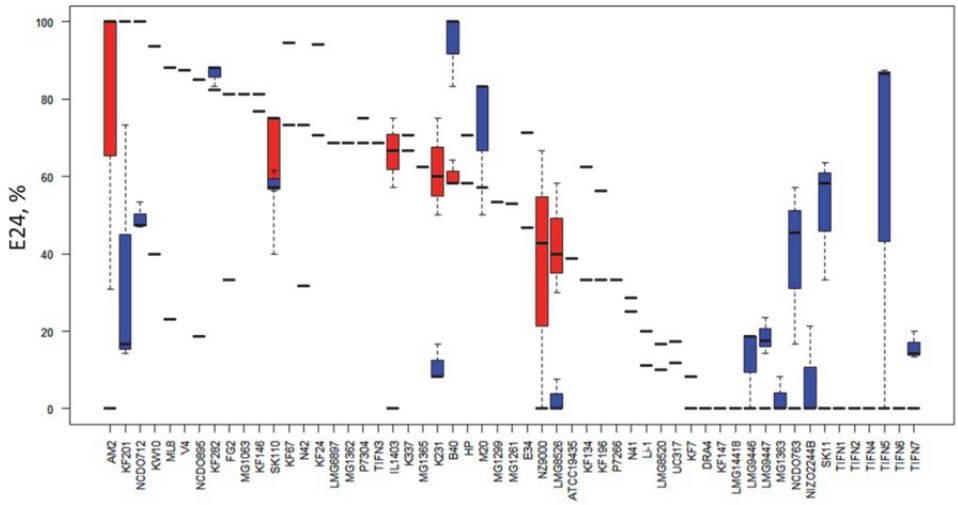

Figure S6. Emulsion stability (E24, %) (y-axis) of *55 Lactococcus lactis* (x-axis) (n=3). Bacterial strains were sorted from the highest to the lowest value for cells from stationary growth phase (shown in red). The values measured for cells from exponential growth phase are shown in blue.

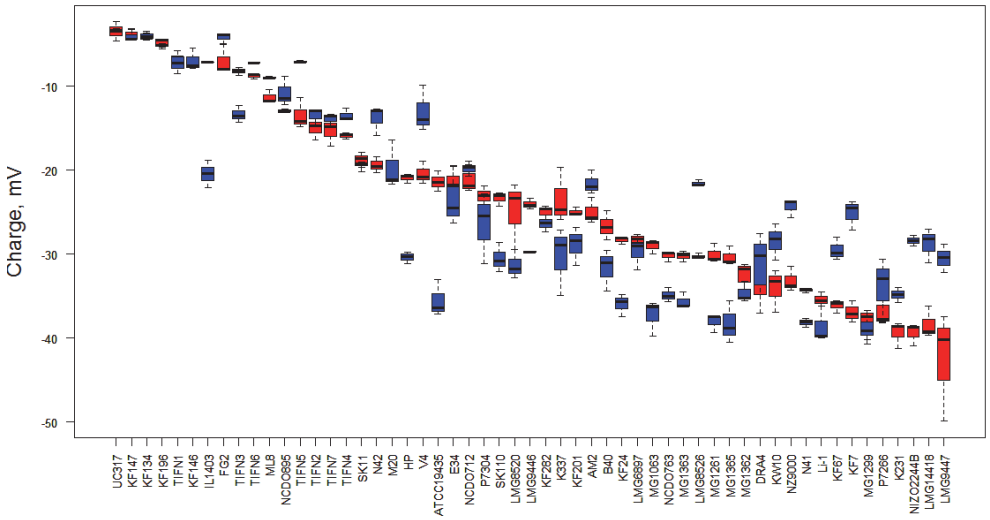

Figure S7. Charge (mV) (y-axis) of *55 Lactococcus lactis* (x-axis) (n=3). Bacterial strains were sorted from the highest to the lowest value for cells from stationary growth phase (shown in red). The values measured for cells from exponential growth phase are shown in blue.

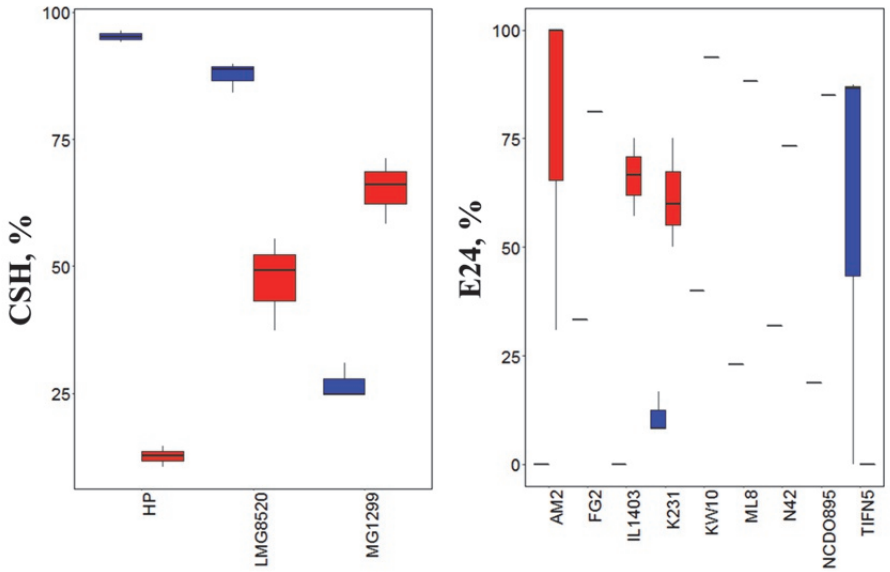

Figure S8. *Lactococcus lactis* (x-axis) with opposite cell surface hydrophobicity (CSH, %) (y-axis) and emulsion stability (E24,%) (y-axis) when originating either from exponentially growing (blue) or stationary cultures (red) (n=3).

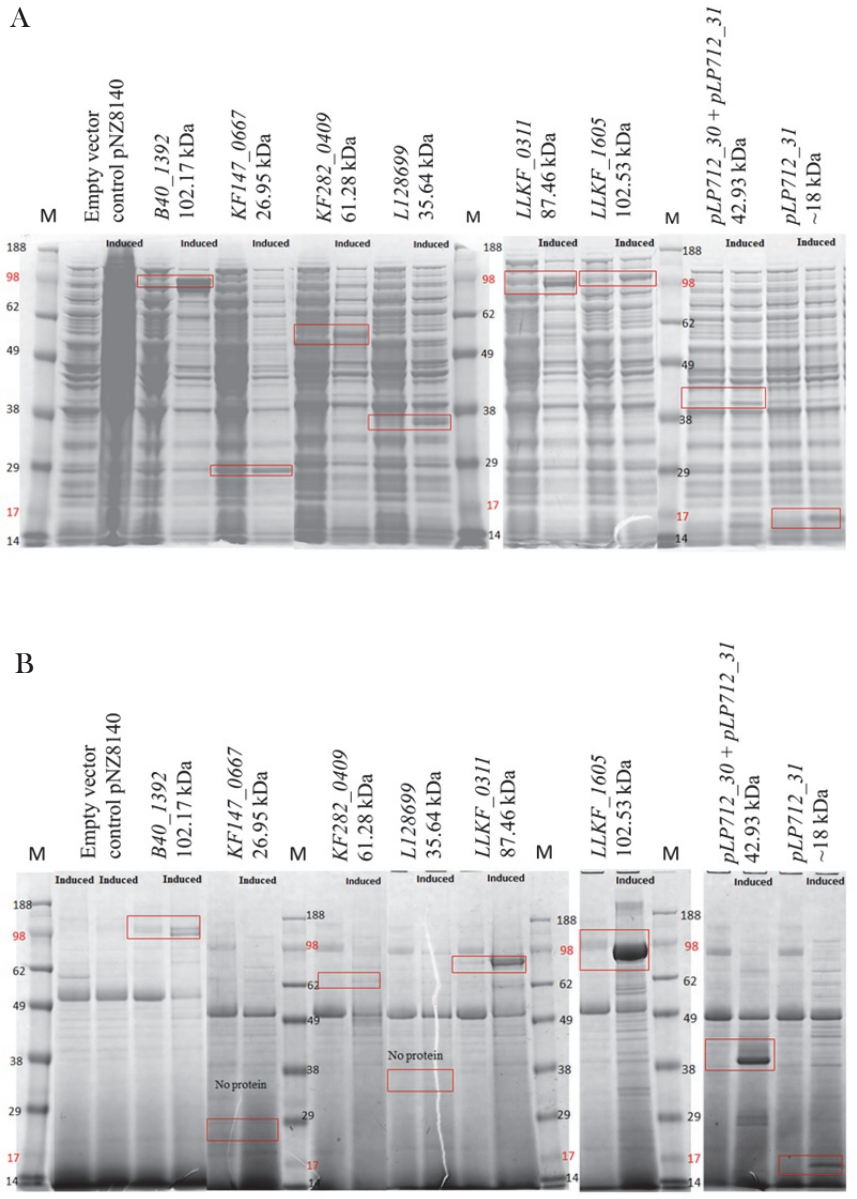

Figure S9. SDS-PAGE gels of the 6 overexpressed proteins in *L. lactis* NZ9000 after induction of exponentially growing cells with 10 ng/mL nisin and incubation for another 5 h. Overexpressed proteins were isolated either from cells (Panel A) or from supernatant (Panel B). Bands of overexpressed proteins are indicated in red boxes. M – marker, λ-HindIII.

Table S1. Oligonucleotides used in this study.

Name (a)	Sequence (5' to 3')
A. Primers used for gene overexpression	
OG3116 forw	ATGATAGCGAAGAACCCTAACACACCAGC
OG3116 revXbaI	GGCTCTAGATAATCACGTTTAGGCTTGCGTTTAACG
OG2970forw	ATGACGAAAGAAAATATCGCGAACTTGGC
OG2970revXbaI	CCGTCTAGACTACAATTCCCCAATTATATTTTCCCATTC
OG2478forw	ATGAATAAGAAAACCCTTATCTCGTCAC
OG2478revXbaI	CCGTCTAGAGTTTCCACATTTTCCATAAATCTATTATATCG
OG1984forw	ATGCGTAAATTGGAAAAATTGTTGAATGACTTGACG
OG1984revXbaI	CCGTCTAGACAAGTCTGCTCTTTTCTATCCTTCTTC
OG1874forw	ATGAAAAAATCGAAAAAATTTTTTATTAGAATATGCCTTTCTACAGGAATTTTGGC
OG1874revXbaI	CCGTCTAGAGGCAAACTTTTACAGCCCTTTGTCAGC
OG2225forw	ATGAAAAATAAAATTATCGTAGTAGCTATACTAATAACAAC
OG2225revXbaI	GGCTCTAGATTAATTTGTGGTTTCTACGGGGCTTAAATAAGC
B. Oligonucleotides for amplification of sequences used to generate double cross-over mutants	
OG2161LFforwXbaI	CCGTCTAGAGAGTACGAACGTCAAACTGAGCAGC
OG2161LFrev + vlag	GCTCTAGGATGTTCATAGGCATAATATTTGCCCAGGTAACATCCAGAAGAAATTACCTAT
OG2161RFforw	GCAAATATTATGCCTATGAACATCCTAGAGC
OG2161RFrevEcoRI	GGCGAATTCGGCTCGATAACCATAAGTTGGATTCCC
OG2161DCOforw	GGACGAAGTGGGTTTGCCTTTTGC
OG2161DCOrev	GTTGAGCTTGTTGGCTATTGAGTGC
OG1903LFforwXbaI	CCGTCTAGACGTCATTAGCACAGTCTTACTAGCAACC
OG1903LFrev + vlag	GACCTAAGATGAAGAGCAACCAAGTCCGGTTTGACTAAAAGCAACTTGTGCACCTCC
OG1903RFforw	GGACTTGGTTGCTCTTCATCTTAGGTC
OG1903RFrevSacI	GGCGAGCTCGAGAGCTTCCTTACTTTAGCTGATGG
OG1903DCOforw	CGGCAACTAATCCACTCACACCTGG
OG1903DCOrev	CCAGCTCGTGACCTTGGTCCTCGC
OG1859LFforwXbaI	GCCTCTAGAGGTTTCGCTTGCTCCAGGATTAGG
OG1859LFrev + vlag	CGCTCCAGATACAATAAAAACAAGAATGACCCCTTCAAGTACCGCTTCTATACTAAAATCAC C
OG1859RFforw	GGTCATTCTTGTTTTTATTGTATCTGGAGCG
OG1859RFrevEcoRI	CCGGAATTCGCAGGTTTGTTCCTGTTCCCGTTCCTGC
OG1859DCOforw	GACCCAACGACTAAAGCGCAGACAC
OG1859DCOrev	GAGTGCCAGCAATGTCAAAGTATCACC
srtALFforwBamHI	CCCGGATCCGGATAAAGAAGCAGCAGTTGATAAAGCC
srtALFrev + vlag	CACAGAATCTGTCACTTTATCAAATGCCTTCTCGCGCTCTTCCATCTATATATGACCACC
srtARFforw	GAGAAGGCATTTGATAAAGTGACAGATTCTGTG
srtARFrevEcoRI	GGCGAATTCCGCAAAATAAGCAAGAGAATCTTCATCTCC
srtADCOforw	GGTGACGGTTTATCAACCATGATTTATGC
srtADCOrev	CCAAAAGCTTGGCCGCAGTTTCTTGCC

(a) The letters "rev" and "forw" indicate oligonucleotides annealing to the DNA strand in reverse or forward direction, respectively. XbaI, EcoRI, SacI, BamHI indicate restriction sites within the sequence. LF and RF indicates if the oligonucleotide is located upstream or downstream of the amplified gene and the annotation +vlag indicates nucleotides in a "LF" amplicon that overlaps with the "RF" amplicon which allows to perform a SOE-PCR. The letters DCO indicate oligonucleotides used to confirm double crossover mutants.

Table S2. Cell surface properties of *55* lactic acid bacteria (n=3). Surface properties were measured at exponential and stationary growth phases. We measured the cell surface hydrophobicity (CSH, %) to two hydrocarbons – petroleum (PCSH, %) and hexane (HCSH, %), emulsion stability (E24, %), charge (ZP, mV), attachment to milk proteins: para-caseinate (ParaCN, %), sodium caseinate (NaCN, %) and sodium caseinate heated for 10 min at 90°C (NaCN90C, %). The values represent the (average ± standard deviation) calculated for 3 biological replications.

Strain	Growth phase	ParaCN, %	NaCN, %	NaCN90C, %	PCSH, %	E24P, %	HCSH, %	E24H, %	ZP, mV
AM2	Exponential	95.7±0.9	96.8±0.6	95.9±1.2	4.1±2.4	0±0	2.3±0.6	0±0	-21.6±1.4
ATCC19435	Exponential	1.5±1.3	1.4±1.2	0.8±0.3	19.6±3.7	38.9±0	22±1.4	35±0	-35.5±2.2
B40	Exponential	97.5±0.2	97.7±0.4	97.9±0.8	51.2±5.2	94.4±9.6	52.3±3.7	94.4±9.6	-31.6±2.5
DRA4	Exponential	92.8±1.1	93.9±1.3	86.3±11.4	11.4±0.4	0±0	12.3±1.5	0±0	-31.6±4.9
E34	Exponential	37.3±2.4	49.1±1.5	57.7±1.7	79.1±3.6	71.4±0	86.8±1	56.3±0	-23.4±3.6
FG2	Exponential	94.2±1.7	96.1±0.5	94.8±2.1	48.7±5.4	33.3±0	71.1±4	38.5±0	-4.2±0.7
HP	Exponential	97.5±1	97.7±0.4	95.4±3.2	95.2±1.1	70.6±0	94.6±0.9	70.6±0	-30.4±0.7
IL1403	Exponential	68.9±2.6	66.3±3	61.7±5.6	8.4±4.3	0±0	2.8±1.4	0±0	-20.4±1.7
K231	Exponential	1.3±0.9	3.1±2.6	2.8±1.3	5.4±0.9	11.1±4.8	4.6±1.4	11.1±4.8	-34.8±0.9
K337	Exponential	1.3±0.8	4.1±5.4	2.1±1	51.8±1.2	70.6±0	54.4±2.4	62.5±0	-30.3±4.1
KF134	Exponential	3±1.9	1.1±0.9	2.8±1	44.7±3.6	62.5±0	46.2±6.9	41.2±0	-4±0.5
KF146	Exponential	57.9±4.4	60.2±1.7	47.9±6.5	67.9±3.8	81.3±0	71.1±3.1	81.3±0	-7±1.3
KF147	Exponential	66.9±1.3	61.2±4.3	62.7±2.7	10±1.2	0±0	7.3±1.3	0±0	-4±0.6
KF196	Exponential	2.7±0.7	7.8±1.7	1.9±0.5	68.5±2.7	56.3±0	51.6±5	58.8±0	-5.2±0.2
KF201	Exponential	0.6±0.4	1.1±0.9	0.6±0.5	66.8±3.7	34.8±33.4	72.4±2.2	34.8±33.4	-28.8±2.3
KF24	Exponential	68.4±1.5	69.4±6.6	62.6±10.2	33.1±2.8	94.1±0	27.3±8.4	94.1±0	-35.9±1.3
KF282	Exponential	0.6±0.3	4.1±6.7	1.4±0.3	76.2±4.1	86.6±2.8	71.7±0.8	86.6±2.8	-26.3±1
KF67	Exponential	66.9±15.2	51.8±10.9	26.8±22.2	26.6±0.4	94.4±0	27.4±0.9	94.4±0	-29.5±1.3
KF7	Exponential	1.6±1	1.1±0.9	1.4±0.5	3.8±2.7	0±0	4.6±0.2	0±0	-25.1±1.8
KW10	Exponential	2.1±0.3	2.5±1.6	1.2±0.8	87.6±3.4	40±0	82±7.4	17.6±0	-28.4±2.2
Li-1	Exponential	3.6±2.3	3.1±4	2.9±3.1	8.6±1.1	11.1±0	9.7±2.8	10.5±0	-38.6±2.1
LMD9	Exponential	93.3±2.9	91.5±3.2	86.2±4.3	45±4.8	53.5±34.3	42.3±3.4	53.5±34.3	-4.6±1.1
LMG14418	Exponential	33±7	35±3.6	29.2±4.2	19.8±7.7	0±0	12.8±1.9	0±0	-28.7±2.1
LMG6897	Exponential	0.4±0.2	2.4±2.3	0.2±0.1	91.4±1.6	68.8±0	93.4±1.7	68.8±0	-29.7±1.9
LMG8520	Exponential	82.8±6.9	65.1±22.7	78.9±6.6	87.6±3	10±0	95.2±0.8	9.1±0	-31.3±1.7
LMG8526	Exponential	2.5±2.8	1.2±1.2	1.1±0.1	4.2±0.2	2.6±4.4	4.1±0.9	2.6±4.4	-21.6±0.4
LMG9446	Exponential	96.5±0.8	96.3±0.7	96.4±0.3	32.3±4.9	12.5±10.8	24.9±1	12.5±10.8	-29.7±0.1
LMG9447	Exponential	1±1.3	1.6±1.8	1.8±2.4	0.8±0.5	18.5±4.7	0.6±0.2	18.5±4.7	-30.5±1.7
M20	Exponential	49.9±8.5	46.2±15.6	35.9±3.9	97.2±1.1	72.2±19.2	93.8±2.7	78.6±0	-19.7±2.9
MG1063	Exponential	92.6±2.3	91.6±2.2	93.9±1.8	9.5±8.2	81.3±0	8.8±1.3	62.5±0	-37.3±2.1

MG1261	Exponential	94.9±1.7	82±1.6	92.6±2.8	12.7±10.3	52.9±0	12.1±7.2	46.7±0	-38±1.1
MG1299	Exponential	73.9±1.8	78.2±3.1	69.2±2.6	26.8±3.6	53.3±0	32.4±5.9	40±0	-38.8±1.6
MG1362	Exponential	94.4±2.5	95.4±0.1	96.3±0.2	12.2±11.3	68.8±0	14.7±14	50±0	-34.6±1.3
MG1363	Exponential	96.6±1.7	98±0.3	95.6±2.4	6.3±3.1	2.8±4.8	3.6±0	2.8±4.8	-35.6±1
MG1365	Exponential	94.7±2.1	95±2.2	93.6±3.1	9±3.1	62.5±0	10.2±1.5	43.8±0	-38.3±2.5
ML8	Exponential	94.9±2.3	95.9±0.5	95.3±1.6	36.1±2.4	23.1±0	45.3±3.8	23.1±0	-8.9±0.1
N41	Exponential	42±4.7	34.5±4.3	30.7±10.5	79.6±7.3	25±0	90.4±3.4	21.4±0	-38.1±0.6
N42	Exponential	0.4±0.3	0.3±0.1	0.8±0.7	94.7±6.2	31.8±0	97.8±1.4	30±0	-13.8±1.8
NCDO712	Exponential	95.9±0.6	95.4±1.3	94.6±1.2	98.7±0.5	49.3±3.5	98.8±0.8	50.1±7.7	-19.9±0.7
NCDO763	Exponential	94.1±1	95.8±0.3	93.3±0.9	43.1±2.6	39.8±20.8	29.6±3	39.8±20.8	-34.9±0.8
NCDO895	Exponential	78±8	75±10	62±12	68±1.4	19±0	82±0.4	22±0.2	-11±1.8
NIZO2244B	Exponential	1.2±0.7	0.8±0.2	0.5±0.2	5.1±0.6	7.1±12.4	4±1.3	7.1±12.4	-28.4±0.7
NZ9000	Exponential	87±7.1	89.8±1.2	89.7±3	26.6±2.5	0±0	25.7±4.8	0±0	-24.3±1.1
P7266	Exponential	88.8±4.4	86.4±2.9	84.8±4.6	17.5±4.2	33.3±0	16.3±2.1	33.3±0	-33.9±3.8
P7304	Exponential	2.4±3.5	1.6±0.6	12.2±17.1	72.6±6	75±0	72±1	52.9±0	-26.4±4.2
SK11	Exponential	93.9±1.6	92.2±0.5	92.3±1.5	6.3±0.7	51.8±16.2	7.8±0.7	51.8±16.2	-19.3±0.3
SK110	Exponential	94.5±2.8	92.9±2.6	94.5±2.6	37±1.5	58.3±2.8	41±0.7	58.3±2.8	-30.5±1.8
TIFN1	Exponential	95.9±1.1	96.8±0.5	92.8±0.5	24.3±3.5	0±0	27.3±1.9	0±0	-7.2±1.3
TIFN2	Exponential	91.7±4.4	93.1±4	92.5±2.8	11.7±8.1	0±0	37±0.9	0±0	-13.5±1.1
TIFN3	Exponential	93±3	91.6±3.3	88.2±4.2	80.1±6.8	68.8±0	80.8±5.9	68.8±0	-13.4±1
TIFN4	Exponential	63±13.5	79.3±8.4	90±3.4	15.1±7.2	0±0	7.2±1	0±0	-13.5±0.8
TIFN5	Exponential	91.1±3.2	96.8±0.9	95.8±1.1	10.8±1.9	58.1±50.3	12.8±2.1	58.1±50.3	-7±0.1
TIFN6	Exponential	94.3±2.1	95.8±0.7	94.4±0.5	25.6±3.7	0±0	24.4±2.7	0±0	-7.2±0.1
TIFN7	Exponential	93.6±3.6	79.4±1.6	95.5±2.1	21.2±1.1	15.9±3.6	21.9±2.8	15.9±3.6	-13.9±0.8
UC317	Exponential	96.5±0.3	96.4±0.6	96.4±0.2	63.2±7.3	17.4±0	77.5±3.3	22.2±0	-3.3±0.1
V4	Exponential	2.4±4	0.4±0.4	1.6±0.5	82±2	87.5±0	56.7±1.2	87.5±0	-13±2.8
AM2	Stationary	94.9±1.2	94.5±0.4	94.9±1.7	3.7±1.2	76.9±40	2.2±0.8	76.9±40	-25±1.6
ATCC19435	Stationary	84.6±4.8	87.6±2.1	90.2±1.5	7.3±1.1	38.9±0	4.4±0.5	35±0	-21.3±1.2
B40	Stationary	98.4±1.1	99.3±0.3	99.2±0.1	52.3±11.1	60.3±3.4	44.6±7.2	60.3±3.4	-26.6±1.8
DRA4	Stationary	89.6±2.2	91.7±1.4	89.4±2	0.5±0.2	0±0	0.3±0.1	0±0	-33.4±3.2
E34	Stationary	15.4±5.9	17.5±5.7	14.8±9.5	93.7±1.7	46.7±0	93.7±1	53.3±0	-21.3±1.6
FG2	Stationary	9.5±3.6	9.1±3.4	91.8±1.4	79.5±5.6	81.3±0	62.5±0.9	68.8±0	-7±1.8
HP	Stationary	95.5±1.9	95.9±1.4	95±1.2	12.6±2	58.3±0	3±0.6	58.3±0	-20.9±0.5
IL1403	Stationary	63.7±1.6	41.6±13.6	67.2±2.2	0.7±0.9	66.3±8.9	4.3±1.2	66.3±8.9	-7.1±0.1
K231	Stationary	9.2±2.4	8.3±3.7	11.2±5	2.9±0.1	61.7±12.6	2.8±0.2	61.7±12.6	-39.4±1.6
K337	Stationary	0±0	45.4±8.3	45.2±16.8	97.3±0.4	66.7±0	93.9±1.9	75±0	-23.4±3.3
KF134	Stationary	51.4±4	52.6±3.2	57.5±2.4	42.1±4	33.3±0	44.1±3	43.8±0	-4.1±0.4

KF146	Stationary	86.7±1.7	82.5±2.8	87±1.4	76.2±0.3	76.9±0	79.5±5.2	87.5±0	-7.1±0.5
KF147	Stationary	50.3±1.2	61.2±2	46.4±10.4	13±3	0±0	10.6±1	0±0	-3.8±0.6
KF196	Stationary	71.2±9.9	68.3±1	69.1±2.1	58.4±3.9	33.3±0	62.5±1.2	53.3±0	-4.8±0.6
KF201	Stationary	8.1±1.5	15.1±7.7	5.1±5.5	94±4.3	100±0	94.2±0.5	100±0	-25±0.5
KF24	Stationary	79.6±5	79.7±2.7	79.9±4.1	14.9±20.3	70.6±0	36.7±0.5	88.9±0	-28.3±0.4
KF282	Stationary	60.7±0.7	63.9±1.7	64.5±0.9	95±1.5	82.4±0	94.3±0.6	82.4±0	-25±0.9
KF67	Stationary	55.8±1.5	61.2±1.7	60.3±2.9	22.1±1.6	73.3±0	22.9±1.3	73.3±0	-36.1±0.8
KF7	Stationary	12.1±11.7	13.8±3.8	1.9±3.2	2.8±0.4	8.3±0	2±0.8	8.3±0	-36.9±1.3
KW10	Stationary	10.9±6.9	11.3±2.1	9.2±4.2	93.5±0.8	93.8±0	96.9±0.7	83.3±0	-34±2.6
Li-1	Stationary	14.8±2.4	18.4±1.7	17.9±6.6	34±7.5	20±0	34.4±3.5	42.9±0	-35.4±0.9
LMG14418	Stationary	31±20.7	13.6±3	23.2±24	16.3±6.5	0±0	11.2±1.9	0±0	-38.3±1.9
LMG6897	Stationary	78.1±0.5	79.7±1.2	79.2±0.8	87±3.7	68.8±0	89.8±1.8	68.8±0	-28.2±0.7
LMG8520	Stationary	70.9±9.2	81.3±2.5	83.8±3.4	47.3±9.2	16.7±0	37.8±17.9	41.4±0	-24.8±4
LMG8526	Stationary	88.4±0.8	91.9±0.1	91.9±0.9	3.7±0.2	42.8±14.4	3.2±0.2	42.8±14.4	-30.2±0.3
LMG9446	Stationary	89.8±1	90.3±2.2	90.9±4.5	13.5±0.4	0±0	13.1±0.3	0±0	-24±0.7
LMG9447	Stationary	86.1±3.9	82.8±4.4	84.9±7.7	1±0.3	0±0	0.9±0.3	0±0	-42.5±6.6
M20	Stationary	86.4±1.7	87.6±3.3	88.6±3	86.8±7.7	57.1±0	94.5±1.4	35.7±0	-20.1±0.6
MG1063	Stationary	83±5.1	89.5±2.6	89.6±2.1	16.7±0	81.3±0	10.3±0.5	62.5±0	-29±0.9
MG1261	Stationary	88.8±6.3	94±1.4	92.5±0.8	21.8±0.2	52.9±0	8.9±1.5	46.7±0	-30±1.2
MG1299	Stationary	79.2±2.2	82.8±2.7	82.7±0.3	65.2±6.5	53.3±0	79±2.4	40±0	-38.3±2.1
MG1362	Stationary	79.6±14.1	89.6±3.4	84.9±9.7	5.6±9.6	68.8±0	9.2±1.6	50±0	-32.6±2
MG1363	Stationary	79.4±1.7	82.3±0.9	82.8±2	5.8±0.2	0±0	8.1±0.2	0±0	-30.2±0.7
MG1365	Stationary	76.6±26.4	92.9±1.8	93.1±1.3	28.6±6.1	62.5±0	12.7±1.3	43.8±0	-30.3±1.2
ML8	Stationary	17.1±6.4	19.8±5.6	13.9±4.6	26.3±12.3	88.2±0	42.1±9.2	88.2±0	-11.3±0.8
N41	Stationary	15.3±10.1	18.1±1.2	17.5±15.2	69.5±9	28.6±0	87.5±5.4	42.9±0	-34.3±0.3
N42	Stationary	90.2±1.9	85.8±3.6	91.8±0.2	96.8±1.6	73.3±0	100±0	33.3±0	-19.4±1
NCDO712	Stationary	94.7±0.6	96±0.6	95.7±2	99.4±0.3	100±0	99±0.6	100±0	-21.1±1.9
NCDO763	Stationary	92.5±1.4	92±1.8	92.6±0.5	28±1.2	0±0	32.5±4.1	0±0	-30.2±0.6
NCDO895	Stationary	81.2±4.1	86.1±5.2	85.2±1.5	75.6±12.5	85±0	94.2±0.3	71.4±0	-12.9±0.2
NIZO2244B	Stationary	41.6±0.8	26.5±15.6	32.2±18.1	7.9±4.1	0±0	1.2±0.5	0±0	-39.4±1.3
NZ9000	Stationary	65.8±23.6	65.8±6.9	65.2±6.9	13.3±2	36.5±33.8	13±2.2	36.5±33.8	-33.1±1.5
P7266	Stationary	0.2±0.4	2.2±3.6	0±0	44.5±3.2	33.3±0	37.5±3.4	33.3±0	-36.8±2
P7304	Stationary	16.6±4.4	18.6±6.3	33.9±29.1	94.7±0.8	68.8±0	94.8±1	37.5±0	-23±1.2
SK11	Stationary	96.6±0.1	97.4±0.3	95.4±1.7	7.8±1.7	0±0	3±1.5	0±0	-18.9±1.2
SK110	Stationary	93±6	97.6±0.6	97.2±1.5	7.5±0.3	63.3±20.2	28.8±1.3	63.3±20.2	-23.3±0.9
TIFN1	Stationary	96.6±0.4	97.5±0.1	97.7±0.3	20.5±0.2	0±0	7.9±0.9	0±0	-6.5±0.1
TIFN2	Stationary	84.2±10.3	91.5±0.6	93.4±2.4	5.3±3.3	0±0	4.2±1.6	0±0	-1.5±1.3

TIFN3	Stationary	82.5±22.5	93.4±1.9	94.2±2.1	68.5±3.8	68.8±0	70.7±6.3	68.8±0	-8.2±0.5
TIFN4	Stationary	78.6±14.5	84.3±8.1	79.3±3.4	1.5±0.6	0±0	1.9±0.5	0±0	-15.9±0.4
TIFN5	Stationary	98.6±0.7	98.3±0.3	98.1±0.8	4±1.6	0±0	2.7±0.3	0±0	-13.4±1.9
TIFN6	Stationary	80.4±25.1	97±0.8	96.8±0.7	3.1±0.6	0±0	3±0.5	0±0	-8.8±0.3
TIFN7	Stationary	92.6±1.6	89.8±0.1	92.9±4.3	2.6±0.3	0±0	2.3±0.2	0±0	-15.3±1.6
UC317	Stationary	88.5±0.4	92.6±0.5	91±3.4	76.6±2.5	11.8±0	63±6.2	36±0	-3.4±1.1
V4	Stationary	22.7±2	12.3±2.7	14.2±4.6	88.8±3.1	87.5±0	69.2±4.6	87.5±0	-20.4±1.3

Table S3. The 18 genes selected for further characterization.

Protein name /locus tag of gene	Locus_tag	Modification	Growth phase [B]	Gene Presence (Pr) or Absence (Ab)	Predicted phenotype change[A]
Cell surface protein precursor/ *B40_0084*	B40: locus_tag=B40_0084	Overexpression in MG1363 from B40 (930 aa)	ST	Pr	ZP ▼
Cell wall surface anchor family protein/ *pLP712_21*	MG1299: locus_tag=pLP712_21	Overexpression in MG1363 from pLP712 of MG1299 (371 aa)	EX	NA	CSH ▲
Ribose 5-phosphate isomerase A/ *KF147_0667*	KF147 locus_tag=KF147RAST_0688	Overexpression in MG1363 from KF147 (234 aa)	EX	Ab	ParaCN ▼
Possible surface protein/ *LLKF_0684*	Locus tag in KF147: LLKF_0684	Overexpression in MG1363 from KF147 (999 aa)	ST	Pr	ZP ▼
Cell wall surface anchor family protein/ *llmg_1148*	MG1363: locus_tag=llmg_1148; NZ9000: locus_tag=LLNZ_05925	Knock-out in MG1363 (567 aa)	ST	Ab	ZP ▼
Iinternalin, putative (LPXTG motif)/ *IL1403RAST_1416*	IL1403: locus_tag=IL1403RAST_1416	Overexpression in MG1363 from IL1403 (653 aa)	EX	Pr	CSH ▲
Internalin, putative (LPXTG motif)/ *KF282_0409*	KF282: locus_tag=0409	Overexpression in MG1363 from KF282 (559 aa)	ST	Ab	ZP ▼
Cell wall surface anchor family protein/ *llmg_0009*	MG1363RAST_0009 locus_tag=llmg_0009	Knock-out in MG1363 (227 aa)	EX	Ab Ab	ParaCN ▼ CSH ▲
Hypothetical protein/ *L128699*	Locus tag: IL1403RAST_1821	Overexpression in MG1363 from IL1403 (yreB, 314 aa)	EX	Pr	CSH ▲
Hypothetical protein/ *D688_p3025*	MG1363: locus_tag=D688_p3025	Knock-out in MG1363 (TraD, 612 aa)	EX	Ab	ParaCN ▼
Cell wall surface anchor family protein/ *LLKF_0311*	KF147: locus_tag=LLKF_0311	Overexpression in MG1363 from KF147 (809 aa)	EX	Pr Ab	CSH ▲ E24 ▲
Xyloside transporter/*XynT*	IL1403 : locus_tag=L0233	Overexpression in MG1363 from IL1403 (490 aa)	ST	Ab Ab	ParaCN ▲ NaCN ▲
Cell surface protein precursor/ *llmg_1096*	MG1363: locus_tag=llmg_1096; NZ9000: LLNZ_05670; MG1299~llmg_1096	Knock-out in MG1363 (387 aa)	ST	Ab	ZP ▼
Extracellular protein/ *llmg_1095*	IL1403: locus_tag=L191998; NZ9000~LLNZ_05665; MG1299~llmg_1095; MG1363: locus_tag=llmg_1095	Knock-out in MG1363 (247 aa)	ST	Ab	ZP ▼
endo-beta-N-acetylglucosaminidase / *LLKF_1605*	KF147 locus_tag=LLKF_1605	Overexpression in MG1363 from KF147 (923 aa), gene="*ypcCD*"	ST	Ab Ab	ParaCN ▲ NaCN ▲
Hypothetical protein/ *llmg_1093*	IL1403: locus_tag=193176; MG1363: locus_tag=llmg_1093; NZ9000~LLNZ_05655; MG1299~llmg_1093	Knock-out in MG1363 (334 aa)	ST	Pr	NaCN ▲
Sortase A/*srtA*/ *llmg_1449*	locus_tag=llmg_1449	Knock-out in MG1363 (250 aa)		Manual selection	NA
Sortase C/ *srtC*/ *llmg_1801*	locus_tag=llmg_1801	Knock-out in MG1363		Manual selection	NA

[B] Data for genotype-phenotype matching was obtained from cells that originated from the indicated growth phase; EX-Exponential growth phase, ST –stationary growth phase.

[A] ZP – charge (mV), ParaCN – attachment to para-caseinate (%), NaCN – attachment to sodium caseinate (%), NaCN90C – attachment to sodium caseinate heated at 90°C for 10 min (%), CSH – cell surface hydrophobicity (%), E24 – emulsion stability for 24h (%), NA – not applicable, ▲ – cell surface property increases, ▼ – cell surface property decreases.

Table S4. Cell surface properties of the 10 obtained mutants. The measured properties are cell surface hydrophobicity (CSH, %) measured with petroleum, emulsion stability after 24 h (E24, %), charge (ZP, mV), attachment to sodium caseinate (NaCN, %), to the same protein heated for 10 min at 90°C (NaCN90C, %), and to para-caseinate (ParaCN, %). The results represent the average of 6 measurements and standard deviation. Significance was calculated using a two-tailed distribution t-test comparing surface properties of each strain to its control in the corresponding growth phase; * $p<0.01$

Strain	Protein	ParaCN, %	NaCN, %	NaCN90C, %	CSH, %	E24, %	ZP, mV
Overexpression in *L. lactis* NZ9000pNZ8150							
Exponential growth phase							
NZ9000pNZ8150		98.9±0.5	99.2±0.5	98.9±0.4	11.1±13.9	0±0	-29.3±2.4
NZ9000pNZ8150_*B40_0084*	Cell surface protein precursor	99.3±0.6	98.9±1.1	99.3±0.3	23.7±26.0	0±0	-25.3±1.5
NZ9000pNZ8150_*KF147_0667*	Ribose 5-phosphate isomerase A	98.1±1.5	98.9±0.3	98.7±0.5	4.1±1.8	0±0	-27.5±1.1
NZ9000pNZ8150_*KF282_0409*	Internalin_putative_LPXTG motif	96.3±4.6	94.2±11.6	95.5±5.6	49.6±6.3	8.3±20.4	-24.7±1.9
NZ9000pNZ8150_*L128699*	Hypothetical protein	98.9±0.8	99.2±0.4	98.3±2.5	2.6±0.9	0±0	-26.6±2.5
NZ9000pNZ8150_*LLKF_0311*	Cell wall surface anchor family protein	32.4±50.3*	43.1±47.6*	38.6±48.3*	40.7±35.9	0±0	-25.2±1.7
NZ9000pNZ8150_*LLKF_1605*	Endo-beta-N-acetylglucosaminidase	98.5±1.0	98.8±0.5	99.2±0.6	2.4±2.3	0±0	-26.5±1.0
Stationary growth phase							
NZ9000pNZ8150		97.9±0.1	98.7±0.6	97.9±0.7	8.8±5.0	0±0	-27.4±1.5
NZ9000pNZ8150_*B40_0084*	Cell surface protein precursor	98.9±0.8*	99.8±0.1*	99.7±0.2*	89.4±6.8*	58.6±25.9	-25.1±1.3
NZ9000pNZ8150_*KF147_0667*	Ribose 5-phosphate isomerase A	99.1±0.5*	99.5±0.1*	99.5±0.2*	4.8±2.6	0±0	-27.8±3.6*
NZ9000pNZ8150_*KF282_0409*	Internalin_putative_LPXTG motif	99.1±0.3*	94.5±11.9*	99.3±0.7*	49.3±21.8	31.4±35.1	-27.1±1.6
NZ9000pNZ8150_*L128699*	Hypothetical protein	98.9±1.0*	98.7±1.1*	98.3±1.8*	2.4±4.5	0±0	-28.2±0.7
NZ9000pNZ8150_*LLKF_0311*	Cell wall surface anchor family protein	31.2±48.4*	29.9±46.4*	31.5±48.8*	78.3±9.0*	0±0	-26.8±0.7
NZ9000pNZ8150_*LLKF_1605*	Endo-beta-N-acetylglucosaminidase	99.5±0.3*	99.5±0.3*	99.6±0.1*	23.7±16.1	0±0	-27.3±0.9
Knock-out in *L. lactis* MG1363							
Exponential growth phase							
MG1363		96.6±1.7	98.0±0.3	95.6±2.4	6.3±3.1	0±0	-25.6±0.9
MG1363Δ*llmg_1383*	Conjugal transfer protein (TraG)	41.9±6.4	48.3±9.6	42.8±4.7	7.7±2.5	0±0	-29.1±0.7
MG1363Δ*llmg_1096*	Cell surface protein precursor	98.6±0.4	98.9±0.5	98.9±0.3	6.9±4.2	0±0	-27.8±0.4
MG1363Δ*llmg_1093*	Hypothetical protein	25.5±22.3	55.9±35.0	28.9±25.2	1.2±2	0±0	-28.8±0.5
MG1363Δ*llmg_1449*	Sortase A	98.3±0.6	98.9±0.2	97.5±1.6	7.9±2.8	0±0	-29.9±1.1
Stationary growth phase							
MG1363		79.4±1.7	82.3±0.9	82.8±1.9	5.8±0.2	0±0	-30.2±0.7
MG1363Δ*llmg_1383*	Conjugal transfer protein (TraG)	95.9±3.9	85.5±11.9	97.7±0.7	0.9±1.6	0±0	-28.8±2.5
MG1363Δ*llmg_1096*	Cell surface protein precursor	98.8±0.4	99.4±0.1	99.2±0.4	5.3±4.9	0±0	-29.3±0.9
MG1363Δ*llmg_1093*	Hypothetical protein	47.5±48.9	25.4±22.4	43.6±39.7	6.9±6.7	0±0	-29.5±1.8
MG1363Δ*llmg_1449*	Sortase A	98.8±0.5	99.1±0.4	99.2±0.1	0.5±1.3	0±0	-28.9±0.9

Chapter 4

Altering textural properties of fermented milk by using surface-engineered *Lactococcus lactis*

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Abstract

Lactic acid bacteria are widely used for the fermentation of dairy products. While bacterial acidification rates, proteolytic activity and the production of exopolysaccharides are known to influence textural properties of fermented milk products, little is known about the role of the microbial surface on microbe-matrix interactions in dairy products. To investigate how alterations of the bacterial cell surface affect fermented milk properties, 25 isogenic *Lactococcus lactis* strains that differed with respect to surface charge, hydrophobicity, cell-chaining, cell-clumping, attachment to milk proteins, pili expression and EPS production were used to produce fermented milk. We show that overexpression of pili increases surface hydrophobicity of various strains from 3-19% to 94-99%. A profound effect of different cell surface properties was an altered spatial distribution of the cells in the fermented product. Aggregated cells tightly fill the cavities of the protein matrix, while chaining cells seem to be localized randomly. A positive correlation was found between pili overexpression and viscosity and gel hardness of fermented milk. Gel hardness also positively correlated with clumping of cells in the fermented milk. Viscosity of fermented milk was also higher when it was produced with cells with a chaining phenotype or with cells that overexpress exopolysaccharides. Our results show that alteration of cell surface morphology affects textural parameters of fermented milk and cell localization in the product. This is indicative of a cell surface-dependent potential of bacterial cells as structure elements in fermented foods.

Keywords: *Lactococcus lactis*, microbe-matrix interactions, cell surface, EPS, pili, viscosity, gel hardness.

Introduction

Lactic acid bacteria (LAB) are Gram-positive bacteria that are generally regarded as safe (GRAS) and are used extensively in food and feed fermentations. They are also found on mucosal surfaces of humans and animals (1, 2). One of the dominant attributes of LAB is the fact that they produce lactic acid as the main metabolic end product of fermentation, which leads to acidification and preservation of the fermented product. An additional functionality of many strains is their ability to produce volatile metabolites that are important flavour compounds (3–5). LAB can also play a significant role in altering textural properties of the end products through acidification, proteolytic activity or the production of extracellular polysaccharides (EPS) (6). One economically very important species of LAB, *Lactococcus lactis*, is used worldwide in the dairy industry for the production of quark, buttermilk and a huge variety of cheeses.

In general, the matrix of fermented dairy products like yoghurt and cheese consists of aggregated caseins, whey proteins and fat droplets, interspersed with serum and/or whey pockets. In addition, it contains minerals, salts, and microorganisms. Interactions between milk components in the matrix and their effect on functionality have been studied extensively (7–11). For example, apart from protein-protein interactions, the rheological properties of a milk gel also depend on the size and number of fat droplets and their surface composition (12). When milk is homogenized, the fat droplets are covered and stabilized by milk proteins. Because of the cross-linking interactions between proteins covering the fat droplets, the milk gel is strong and has a low flow and enhanced stability against the expulsion of serum from the contracting protein matrix, syneresis. Interactions between constituents in the food matrix can be hydrophobic (13), electrostatic (14), based on hydrogen bonding (15), Van der Waals, depletion interaction (16), the consequence of steric repulsion (17) and/or caused by salt bridges (18).

Relative to the large body of knowledge on interactions between the various milk components (19, 20), little is known about microbe-matrix interactions and the possible interactions between LAB and matrix components of fermented products (21–24).

Interactions between microorganisms and milk components could occur via surface properties of both particles (25, 26), while different types of interactions can occur at the same time, such as Van der Waals interactions, electrostatic repulsions and attractions, hydrogen bonds, hydrophobic interactions, salt bridges, and steric interactions (27–29). The surface properties of bacteria are determined by the molecular composition of their cell walls, which can be decorated with (lipo-)teichoic acids, proteins (30), pili, or capsular polysaccharides (CPS) (31–33).

The biodiversity of *L. lactis* surface properties is very high (34), (see also Chapter 3). Pili can significantly change the surface of *L. lactis* upon their overexpression (35), (Chapter 2). Large surface changes can also be introduced by plasmid transfer through conjugation (36–38), or by altering the expression of genes such as those encoding autolysins (39, 40) or enzymes involved in galactose utilization (41). The molecular composition of the cell wall has a big impact on the roughness of the bacterial surface, on bacterial chaining and on cell aggregation (35), (see also Chapter 2). These properties govern the interactions between LAB and the matrix components (24, 26). The production of EPS by a starter culture is known to affect textural properties through the binding of water and thereby increasing milk ropiness. This leads to an increase in milk viscosity and a reduction of syneresis (42–44). The charge, stiffness, and linearity of EPS molecules impact on rheological and physical properties of the fermented milk matrix. EPS modification by partial removal of side groups leads to a reduction of efficiency as a thickener (45).

Contrary to the role of EPS on textural properties of fermented milk, little is known about the influence of bacterial surface properties on interactions with the matrix and their functional consequences on flavour and texture (26, 46, 47). More specific interactions between *L. lactis* flavours, milk proteins and emulsions were studied by Ly *et al.* (48, 49). Here, we studied how bacterial surface properties impact textural parameters of fermented milk. For this, we used 25 isogenic *L. lactis* strains which differed in known surface properties and cell morphologies. We found that particular surface alterations lead to distinct differences in gel hardness and viscosity of fermented

milk. Based on our results we propose that bacteria can be used as structure elements in fermented foods.

Materials and methods

Bacterial strains and growth conditions

L. lactis strains (Table 1) were grown at 30°C in M17 broth (Oxoid Ltd, Basingstoke, UK) containing 1% glucose (GM17) or 1% lactose (LM17). When required, erythromycin (Ery; 10 μg/mL), chloramphenicol (Cm; 5 μg/mL), rifampicin (Rif; 50 μg/mL), or streptomycin (Str; 100 μg/mL) were added to the indicated final concentrations.

Cell surface properties

Bacterial cell surface properties, i.e., surface charge (or zeta potential, ZP, mV), cell surface hydrophobicity (CSH, %), emulsion stability after 24h (E24, %) and attachment to milk proteins were measured as described earlier (34) (Chapter 3).

Preparation of fermented milk

Full-fat pasteurized and homogenized milk (3.6% fat, 3.5% protein, 4.7% lactose) was purchased from a local supplier and sterilized at 115°C for 15 min to denature the whey proteins (50). Sterilized milk was supplemented with sterile 50% glucose solution (4% final concentration) and sterile 20% Bacto™ Casitone (Pancreatic digest of casein, BD, Sparks, MD, USA) solution to 0.2% final concentration to ensure growth of lactose-negative and protease-negative strains, respectively. Instead, for lactose-positive and protease-positive strains, a same volume of water was added to the milk to ensure that protein and fat content of the milk were the same for all strains. For gel strength and viscosity measurements on the fermented milk samples, the bacterial strains were pre-cultured overnight in milk supplemented with the appropriate antibiotic, but the antibiotic was not added during the actual milk fermentation.

Warm (30°C) milk was inoculated with 2% v/v of an overnight culture. Acidification rates and final pH were measured with a Cinac 14 ph+2T (Alliance instruments, Freppilon, France). pH electrodes were inserted into the inoculated milk samples in 10 mL tubes and measurement was done every 6 min for 21 h at 30°C.

Gel strength of fermented milk

Aliquots (300 mL) of milk without antibiotic were inoculated with 2% of the strains to be tested. The prepared milk was distributed to three of 100 mL sterile glass cups (70 mm diameter), which were incubated statically for 21 h at 30°C. Gel strength was measured with a Texture Analyzer (TA.XTplus, Stable Micro Systems Ltd., Sprundel, NL) equipped with the 5 kg load cell according to the manufacture application guidelines. The 100 mL fermented milk samples were compressed uniaxially to a depth of 20 mm with a constant speed of 1 mm s^{-1} by a probe with a grid-like geometry having 10 mm side squared openings. The peak force applied on the sample corresponds to the hardness of the milk gel.

Viscosity of fermented milk

After the texture analysis, the viscosity of the fermented milk was measured with a rotational viscometer (Haake Searle RV20 Rotovisco and RC 20 Rheocontroller, ThermoScientific, Hofheim, Germany) with MV2P (medium viscous profile) rotor according to the manufacture guidelines. Fermented milk (60 g) was transferred to the MVP cup and allowed to rest for 15 min. Subsequently, the sample was measured as the shear rate gradually increased from 0 to 400 s^{-1} for 5 min and then decreased again from 400 to 0 s^{-1} for 5 min at 21°C. Viscosity values were determined at a shear rate of 10 s^{-1} of the first curve, i.e., when shear rate was increasing.

Confocal laser scanning microscopy (CLSM)

To one mL of homogenized sterilized milk, with sugar and antibiotics as required, 15 µL of a solution containing 0.5% (final concentration) Acridine Orange (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands) and 0.025% (final concentration)

Rhodamine B (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands) in water was added to stain proteins and fat droplets, respectively. This milk was inoculated with 1% v/v of an overnight culture, after which 1 µL Syto 9 (5 mM in DMSO, ThermoFisher) (1 µL /OD_{600} of 1/mL) was immediately added to the sample to stain the bacterial cells. The milk sample was subsequently transferred to a CLSM slide with a cylindrical plastic cup attached to it which was then covered with a lid (51) to prevent evaporation. The sample was incubated for 21 h at 30°C.

CLSM images were taken using a Leica TCS SP 5 confocal laser scanning microscope (Leica Microsystems CMS GmbH, Mannheim, Germany) with Leica application Suite Advanced Fluorescence v. 2.7.3. build. 9723. The Argon laser was used to visualize the Syto 9-stained bacteria, while the DPSS 561 laser was used to visualize the milk protein and fat droplet in the matrix that were stained by the Acridine Orange and Rhodamine B, respectively. The objective lens used was an HCX PL APO 63×/1.2 /water CORR CS.

Results

Surface altered *Lactococcus lactis*

To investigate if changes of bacterial cell surface properties affect the functionality of lactococci in fermented products, we performed assays to assess starter culture functionality in the 25 isogenic *L. lactis* strains (Table 1) that differed in surface charge, hydrophobicity, chaining, clumping, attachment to proteins, pili expression, and EPS production (Table 2).

Table 1. Strains and plasmids used in this study.

No	*Lactococcus lactis* strains	Characteristic	Reference
1	NCDO712	*L. lactis* dairy isolate, *lac*$^+$, contains 6 plasmids - pLP712, pSH71, pSH72, pSH73, pSH74, pNZ712.	(52)
2	NCDO712 (pIL253*pil*)	Ery^R; harboring pIL253 with pilin operon *spaCB-spaA-srtC1-srtC2* from NCDO712	(35)
3	MG1363	Plasmid-cured derivative of *L. lactis* NCDO712	(52)
4	MG1363(pIL253*pil*)	Ery^R; *L .lactis* MG1363 harboring pIL253 with pilin operon *spaCB-spaA-srtC1-srtC2* from NCDO712	(35)
5	MG1363 (pIL253*pil*Δ1)	Ery^R; *L .lactis* MG1363 harboring pIL253 and pilin operon *spaCB-spaA-srtC1-srtC2* from NCDO712 with 1,5-kb internal deletion in *spaCB*	(35)
6	MG1299(pIL253*pil*)	Ery^R; *L. lactis lac*$^+$ derivative of NCDO712 which additionally to pLP712 harboring the pilin operon (*spaCB-spaA-srtC1-srtC2*) from the same NCDO712 strain	(35)
7	MG1299	Derivative of *L. lactis* NCDO712, harbors pLP712; *lac*$^+$	(52)
8	MG1362	Derivative of *L. lactis* NCDO712 (described to harbor pSH72)	(52)
9	MG1063	Derivative of *L. lactis* NCDO712 (described to harbor pSH73 and pSH72)	(52)
10	MG1261	Derivative of *L. lactis* NCDO712 (described to harbor pSH73)	(52)
11	MG1365	Derivative of *L. lactis* NCDO712 (described to harbor pSH71)	(52)
12	MG1614	Str^R and Rif^R; derivative of *L. lactis* MG1363	(52)

13	MG1614_clu$^+$	*Lac*$^+$. Transconjugant of MG1614, harbors pLP712 from NCDO712 and shows clumping phenotype.	(unpublished data)
14	MG1614_clu$^-$	*Lac*$^+$. Transconjugant of MG1614, harbors pLP712 from NCDO712 and show non-clumping phenotype.	(unpublished data)
15	MG1363Δ*acmA*	Derivative of *L. lactis* MG1363 with deletion of *acmA*, which leads to chaining phenotype	(53)
16	MG1363Δ*ahrC*	Derivative of *L. lactis* MG1363 with deletion of *ahrC*	(54)
17	IL1403Δ*acmAacmD*	Derivative of *L. lactis* IL1403 with deletion of *acmAacmD*, which leads to chaining phenotype	(40)
18	IL1403(pIL253*pil*)	Ery^R; Derivative of L. lactis IL1403 harboring pilin operon from pSH74 of NCDO712; shows chaining phenotype and high hydrophobicity	(35)
19	MG1363Δ*dltD*	Derivative of *L. lactis* MG1363 with deletion of *dltD*	(55, 56)
20	MG1363(pIL253)	Ery^R; Derivative of *L. lactis* MG1363 harboring plasmid pIL253	(35)
21	IL1403	Plasmid-free derivative of *L. lactis* IL594	(57)
22	MG1363(pNZ521; pIL253*pil*)	Cm^R and Ery^R; derivative of *L. lactis* MG1363 harboring pNZ521 and pIL253*pil* (*spaCB-spaA-srtC1-srtC2*) from the NCDO712 strain	(35)
23	MG1363(pNZ521)	Cm^R; derivative of *L. lactis* MG1363 harboring proteolytic positive genes on pNZ521	(58)
24	MG1363Δ*galE*	Derivative of *L. lactis* MG1363 with deletion of *galE* leading to chain formation without galactose in a growth medium	(41)
25	MG1363(pNZ4120)	Ery^R; derivative of *L. lactis* MG1363 harboring EPS gene cluster from B40	(59)

Plasmids			
1	pIL253*pil*	Ery^R; 13.1 kb; pIL253 harboring pSH74 pilin operon *spaCB-spaA-srtC1-srtC2* with 300 bp upstream region	(35)
2	pIL253*pilΔ1*	Ery^R; 11.6 kb; pIL253 harboring *spaCB-spaA-srtC1-srtC2* with 1,5-kb internal deletion in *spaCB*	(35)
3	pNZ521	Cm^R; encodes the extracellular serine proteinase (PrtP) from strain SK110	(58)
4	pLP712	The 55.39 kb plasmid encoding genes for lactose catabolism and a serine proteinase involved in casein degradation	(60)
5	pIL253	Ery^R; 4.9 kb; Low copy-number derivative of pAMβ1	(61)
6	pNZ4120	Em^R; pIL253 derivative containing a 17-kb *Nco*I fragment carrying the *eps* gene cluster from NIZO B40	(59)

The 25 strains are variants of the dairy isolate *L. lactis* ssp. *cremoris* NCDO712 and its plasmid and phage-cured derivative MG1363 (52). Deletion of the genes *acmA* (53), *acmAacmD* (40), *dltD* (55) or *galE* (41) led to a cell chaining. The introduction of pNZ4120, a plasmid encoding the EPS gene cluster from *L. lactis* NIZO B40 (59), in strain MG1363, led to EPS production, which is accompanied by a more positive surface charge (see Table 2). We recently isolated strains of *L. lactis* MG1614 in which the protease/lactose plasmid pLP712 from NCDO712 was introduced through conjugation (unpublished data). As described earlier (62), we obtained strains with clumping Clu^+ and non-clumping Clu^- phenotypes among the transconjugants carrying pLP712 (60). Surface characterization of the transconjugants showed that the non-clumping parent strain MG1614 exhibited a hydrophobicity of ≈20±3%, while the hydrophobicity increased to ≈74±4% and ≈90±4% in the Clu^- and Clu^+ strains, respectively (Table 2).

Table 2. Phenotypic characteristics of stationary *L. lactis* strains measured at pH 6.7. Mean values ± standard deviation of biological triplicates (n=3) are shown. ZP - zeta potential (mV), CSH - cell surface hydrophobicity (%), NaCN - cell attachment to sodium caseinate (%), NaCN90C - cell attachment to sodium caseinate heated at 90°C for 10 min (%), ParaCN – cell attachment to para-caseinate (%) and E24 – emulsion stability after 24h (%).

L. lactis	ZP, mV	CSH, %	NaCN, %	NaCN90C, %	ParaCN, %	E24,%
Pili overexpressing strains are chaining and clumping						
IL1403	-7.2±0.5	0±19.9[r]	41.7±13.6	67.2±2.2	63.7±1.6	0±0
IL1403(pIL253*pil*)	-13.9±1.3*	93.2±4.5*	93±2.8*	94±0.4*	91.8±1.8*	88.9±19.3*
MG1299	-30.3±2.1	75.5±3.3	82.8±2.7	82.7±0.3	79.2±2.2	35.6±24.9
MG1299(pIL253*pil*)	-18.1±1.3*	99.1±1.2*	97.5±1.1*	99±0.6*	97±1*	99.3±0.6
MG1363(pIL253)	-26.4±1.8	3.8±6.8	99.2±0.1	99.4±0.3	80.6±6.2	0±0
MG1363(pIL253*pil*)	-11.9±0.2*	94±2.2*	82.8±7.4	81±6.8*	66±16.4	85.2±15*
MG1363(pNZ521)	-31.7±0.8	18.7±10.1	90.3±2.8	88.8±2.4	79.2±9	0±0
MG1363(pNZ521;pIL253*pil*)	-17±1.9*	99±1*	95.4±0.2	96.5±2.5	89.4±3	65.6±29.9*
NCDO712	-20±0.5	99.4±0.3	96±0.6	95.7±2	94.7±0.6	99.7±0.6
NCDO712(pIL253*pil*)	-20.5±1.3	96.3±0.6	85±10.4	84.2±9	92±3.6	100±0
Chaining phenotype[$]						
IL1403Δ*acmAacmD*	-12±0.4*	9.5±4.6	97.2±0.6*	96.2±2.1*	96.4±1.3*	0±0
MG1363Δ*acmA*	-31.4±1.4	15.5±4.5	89±0.7*	87.6±1	83.5±10	0±0
MG1363Δ*galE*	-28.7±0.8	14.9±4.4	94.2±2.1*	95.6±0.5*	37.5±24.6	0±0
MG1363Δ*dltD*	-29.2±0.2	15.4±3.5	84.9±0.6	83.9±3.1	82.4±3.2	0±0
Non-chaining, non-clumping phenotype[$]						
MG1363	-30.2±0.7	5.8±0.2	82.3±0.9	82.8±1.9	79.4±1.7	0±0
MG1261	-30±1.2	21.8±0.2*	93.9±1.4*	92.6±0.8*	88.8±6.3	0±0
MG1063	-29±0.9	16.7±0*	89.5±2.6	89.6±2.1	83±5.1	0±0
MG1362	-31.7±3.3	5.6±9.6	89.6±3.4	84.9±9.7	79.6±14.1	0±0
MG1365	-30.3±1.2	28.6±6.1*	92.9±1.8*	93.1±1.3*	76.6±26.4	0±0
MG1614	-42±2.4	20.4±3.1	97.1±0.8	94.8±4.1	97.7±1.3	0±0
MG1614_clu[-]	-39.4±0.5	73.9±4.1*	96.3±0.9	94.6±0.6	89.4±4.9	0±0
MG1363Δ*ahrC*	-29.5±1.2	79.9±10.7*	84.5±1.1	81±2.7	80.2±1.7	23.8±2.1*
MG1363(pIL253*pil*Δ1)	-16.9±0.9*	79.7±16.9*	66±11*	64.5±14.4	86.3±5.2	97.8±3.9*

Clumping phenotype						
MG1614_clu[-]	-36±0.4	90.2±3.7[*]	81.7±15	58.1±26.3	94.6±0.6	31.3±4[*]
EPS producing[$]						
MG1363pNZ4120	-18.8±2.2[*]	0±23.8[‡]	96.2±1[*]	98.3±0.5[*]	97.4±0.2[*]	0±0

[*] Significant at $p < 0.01$ – comparisons were made to non-surface modified controls.
[$] Comparisons were made to *L. lactis* MG1363 strain except for MG1614_clu[-] and MG1363(pIL253*pil*Δ1) which were compared to MG1614 and MG1363pIL253, respectively.
[‡] Comparisons were made to strains IL1403 and MG1363.
[!]The average values were slightly below zero.

The strains MG1365, MG1362, MG1063, MG1261 and MG1299 differ from their parent NCDO712 by carrying only 1 or 2 plasmids (52). Although originally described as containing 5 plasmids, we recently discovered that strain NCDO712 has 6 plasmids (35) (see also Chapter 2). One of these plasmids, pSH74, carries a *spaCB-spaA-srtC1-srtC2* gene cluster, which encodes proteins that lead to pili formation on the cell surface upon overexpression (35), (Chapter 2). The overexpression of a different types of pili in *L. lactis* is known to cause auto-aggregation (63). Our results show that overexpression of *spaCB-spaA-srtC1-srtC2* leads to an increase in surface hydrophobicity from 3.8±6.8% to 94±2.2% and a decrease of the cell surface charge from -26.4±1.8 to -11.9±0.2 (Table 2). We also distinguish between chaining and clumping phenotypes. Strains exhibiting a clumping phenotype after mild centrifugation (350 g for 2 min) form strong cell aggregates that remain fast sedimenting clumps after pellet re-suspension. Such clumping is mainly seen with pili-overexpressing strains. In contrast, strains with a chaining phenotypes (e.g. strains carrying mutations in *acmA, dltD,* or *galE*) form long chains of cocci that sediment in overnight cultures. However, after centrifugation the cell pellet can be easily re-suspended and cells do not clump. Strain MG1363(pIL253*pil*Δ1) contains the *spa* pilus gene cluster with an internal deletion of 1.5 kb in the *spaCB* gene encoding the pilin tip protein SpaC and a pilus basal subunit SpaB. This strain forms neither chains nor clumps and retained a high cell surface hydrophobicity. This strain produces pili, but they are not attached to the cell surface (35), (Chapter 2). The used strains differ furthermore in their cell surface charge, emulsification properties and the propensity to bind milk proteins (Table 1). This morphologically diverse collection of strains was used for further analysis.

Acidification rates

As the rate by which *L. lactis* acidifies the milk during fermentation and the final pH in the dairy product can influence the textural properties of the latter, we followed the development of pH for all strains during 21 h of milk fermentation (Table 3). It is known that very fast acidification of milk can result in excessive syneresis, whereas very slow acidification leads to the formation of a weaker gel (64, 65). The maximum acidification rate for most strains was ≈0.5 pH/h, but strains showing a chaining phenotype (MG1363Δ*acmA,* MG1363Δ*galE*), the pilin-decorated strain MG1363(pIL253*pil*) and its control strain MG1363(pIL253) exhibited slower acidification rates (Table 3). The acidification rates within each comparison group were almost identical, leaving the surface alteration as the main variable for comparisons. The final pH of all milk samples fermented by derivatives of *L. lactis* MG1363 was 4.25±0.04, with the exception of the proteolytic-positive pilus-harbouring strain MG1363(pNZ521;pIL253*pil*), and strain IL1403 and its derivatives (Table 3), which all exhibited elevated pH values.

Table 3. Textural properties of milk fermented by surface altered lactococci. Mean values ± standard deviation (n=3) are shown for independent biological replications for gel hardness (g), viscosity (mPa·s) and pH.

Strain	Gel hardness (g)	pH	Viscosity at shear rate of 10 s^{-1}, mPa·s	Max acidification (n=1), pH/h
Pili overexpressing strains and their parental control strains without pili (grouped per comparison)				
MG1363(pIL253)[¥]	40.1±2.18	4.29±0.05	722.7±11.3	-0.36
MG1363(pIL253*pilΔ1*)[¥]	45.1±1.13*	4.23±0	1050±19.1*	-0.39
MG1363(pIL253*pil*)[¥]	47.8±1.8*	4.23±0.05	1079.3±58.2	-0.39
MG1363(pNZ521)[¥]	42.8±0.4	4.22±0.05	773±64.3	-0.49
MG1363(pNZ521; pIL253*pil*)[¥]	49.1±2.8*	4.34±0.006	1027.2±30*	-0.46
NCDO712[¥]	43.5±0.6	4.24±0.06	1498±60.6	-0.39
NCDO712(pIL253*pil*)[¥]	42.4±3.2	4.23±0.006	1192±146.2*	-0.34
MG1299	38.59±0.6	4.23±0.05	726.1±68.1	-0.49
MG1299(pIL253*pil*)	38.2±0.6	4.27±0.001	797±54	-0.54

IL1403	37.5±0.64	4.45±0.02	680.1±34.5	-0.53
IL1403(pIL253*pil*)	37±0.7	4.47±0.001	745±78	-0.51
Combinations of EPS and pili/no-pili forming strains				
MG1363(pNZ4120)[¥]	48.9±1.9	4.32±0.006	4556±37.7*	-0.53
MG1363(pNZ4120)+ MG1363(pIL253*pil*)[¥]	51.9±1.1	4.22±0.006	3231±78.9	NA
MG1363(pNZ4120)+ MG1363(pIL253*pilΔ1*)	44.4±3.6	4.19±0.006	2921±277.7	NA
MG1363(pNZ4120)+ MG1363(pIL253)[¥]	46.9±2.9	4.24±0.006	3242.7±396.7	NA
MG1363(pNZ4120)+NCDO712	45.6±6.8	4.18±0.01	2847±117.1	NA
MG1363(pNZ4120)+ NCDO712(pIL253*pil*)	46.97±1.48	4.21±0	2976±105.5	NA
Chaining strains and their parental controls				
IL1403	37.5±0.64	4.45±0.02	680.1±34.5	-0.53
IL1403Δ*acmAacmD*	35.4±0.14*	4.39±0.01	1008.5±109.3*	-0.51
MG1363	43.78±0.89	4.24±0.05	926.1±50.5	-0.53
MG1363Δ*acmA*	41.5±1.08*	4.24±0.05	1034.7±18.9*	-0.42
Transconjugants (clumping/non-clumping)				
MG1614	40±0.43	4.23±0.05	832.5±40.9	-0.54
MG1614_clu[-]	39±1.19	4.27±0.06	739±37*	-0.5
MG1614_clu[-]	41.7±1	4.27±0.05	887.9±28	-0.5
NCDO712 derivatives carrying 1 or 2 plasmids and MG1363 derivatives with single gene deletions				
NCDO712	42.4±1.3	4.24±0.06	867.2±106.1	-0.51
MG1363	43.78±0.89	4.24±0.05	926.1±50.5	-0.53
MG1363Δ*ahrC*	43.8±0.43	4.28±0.03	771±205.5	-0.56
MG1363Δ*dltD*	43.9±0.56	4.24±0.05	891.9±66.9	-0.52
MG1363Δ*galE*	44.71±0.58	4.21±0.05	871.1±181.3	-0.45
MG1063	45.17±0.39	4.19±0.05	986.97±20.7	-0.53
MG1261	41±0.91	4.21±0.05	790.97±20.6	-0.54
MG1362	44.1±0.81	4.2±0.04	945.1±40.9	-0.52
MG1299	38.59±0.6	4.23±0.05	726.1±68.1	-0.49

* Significant at $p < 0.05$ – comparisons were made to non-surface modified controls as described in Table 2.

[¥] For the indicated strains independent repeats of the experiments on different days confirmed the results with slightly different absolute values.

NA – not applicable. For cell mixtures the acidification rate was not determined.

Gel hardness

To determine if cell surface properties influence the rheological parameters of fermented milk, we measured gel hardness and viscosity. The gel hardness of fermented milk prepared with the pili-overexpressing strain MG1363(pIL253*pil*) increased by approximately 46% (p=0.009) compared to milk fermented with the empty plasmid control strain MG1363(pIL253) (Table 3). Similarly to MG1363(pIL253*pil*), the proteolytic-positive variant overexpressing pili, strain MG1363(pNZ521; pIL253*pil*), also increased the fermented milk gel strength, by 15% (p=0.04). For three other strains, NCDO712(pIL253*pil*), MG1299(pIL253*pil*), and IL1403(pIL253*pil*), gel hardness did not change significantly compared to their control strains. The fact that IL1403(pIL253*pil*) did not change gel strength can be explained by the very low number of pili on the surface of the cells as compared to MG1363(pIL253*pil*) (35), (Chapter 2). The data suggests that the overexpression of pili has a profound effect on gel hardness, but the effect seems to be strain dependent. When chaining or clumping cells were used gel hardness was significantly decreased. The chain-forming strains IL1403Δa*cmA*a*cmD* and MG1363Δ*acmA* decreased gel hardness compared to their controls by 5.65% (p=0.005) and 5.2% (p=0.049), respectively. Together, these results show that microbial cell surface alterations brought about by either pili overexpression or cell chaining significantly alters the gel hardness of fermented milk.

Viscosity was increased by 19-35% for milk fermented with the strains overexpressing pili, MG1363(pIL253*pil*) (p=0.0005) and MG1363(pNZ521; pIL253*pil*) (p=0.003), relative to their controls (Table 3). In comparison to milk fermented with the EPS producing strain MG1363pNZ4120 (392% increase compared to MG1363, p=0.0007) the increase in viscosity caused by pili production is considerably lower. We did not observe synergistic effects when milk was fermented with a 1:1 mixture of EPS- and pili-producing strains (Table 3).

The use of chaining phenotypes lead to milk gels with increased viscosity. For example, the chaining strains MG1363Δ*acmA* and IL1403Δ*acmA*a*cmD* formed fermented milk

with a viscosity that was 11% (p=0.025) and ≈ 50% (p=0.026) higher compared to the control strains MG1363 and IL1403, respectively (Table 3). Overall, the results of milk viscosity measurements show that the engineering of the surface morphology of *L. lactis* increases product viscosity by up to 50%.

Localization of cells in fermented milk

To investigate if alterations of the bacterial cell surface affect the location of cells in the fermented milk matrix, we performed CLSM imaging on undisturbed fermented milk samples. Due to the sedimentation of cells during fermentation and the limited depth at which CLSM allows imaging (≈40 μm), the number of cells in the image might be higher than in the average sample. Nevertheless, we consider the observed effects to be indicative for what occurs throughout the product and the CLSM images to provide information on the behavior of the bacteria *in situ*. The results (Fig. 1) reveal obvious differences between various strains. For example, loose cocci and clumping cocci locate in the protein matrix close to serum regions (Fig. 1A and C), aggregated cells of MG1363(pIL253*pil*) fill the serum regions between the aggregated proteins in the fermented milk (Fig. 1B), chaining cells are located in protein and serum regions (Fig. 1D and E), while EPS producing cells locate predominantly in serum regions (Fig. 1F). Interestingly, the localization effect observed for MG1363(pIL253*pil*) was not seen for strain IL1403(pIL253*pil*). This would be consistent with the fact that pilin overexpression results in much fewer pili on the surface of IL1403 as compared to MG1363 (35), (Chapter 2) and the limited effect on gel hardness presented above. Overall the CLSM results indicate that surface properties of *L. lactis* can have profound effects on the location of the cells in the fermented milk matrix.

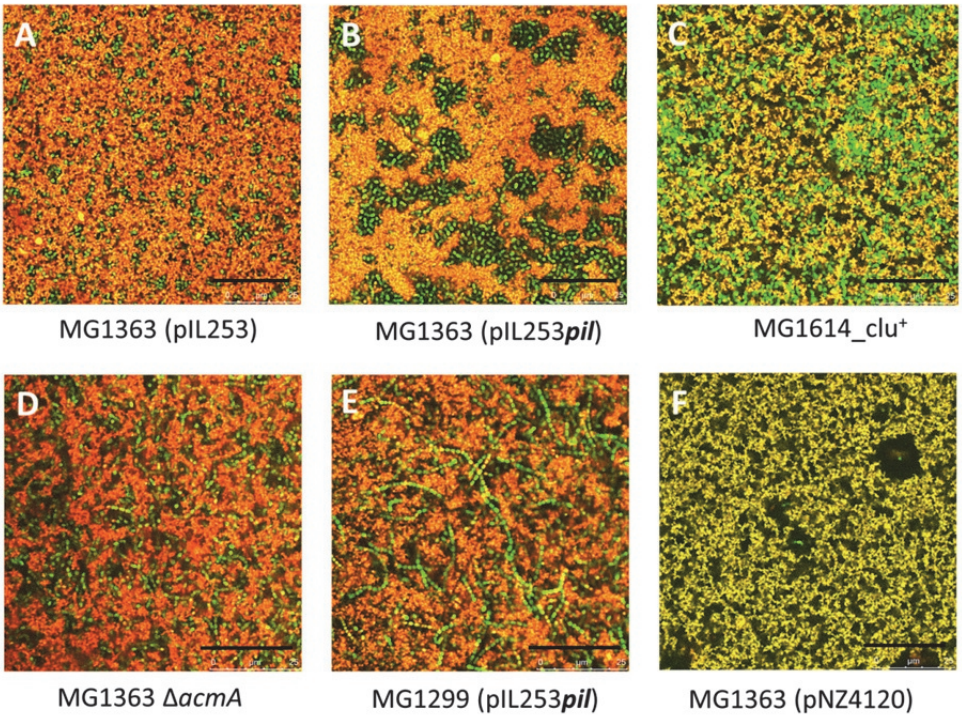

Figure 1. Microstructure of milk fermented by *Lactococcus lactis* strains with altered surface properties. Bacterial cells are green, while proteins and lipids appear orange/red; the black areas represent the serum fraction. The black bar in the right corner indicates 25 μm.

Discussion

Important physical properties of fermented milk are gel hardness and viscosity. It is known that the hardness of fermented milk increases with a higher dosage of casein (66), upon heat treatment (67) or homogenization of milk (68), decreasing pH (69), longer acidification time or rate (70) or lower post-incubation temperature (71). The viscosity of fermented milk increases when the gel is allowed to harden before stirring, when stirring intensity is lowered or when the bacterial strains applied produce EPS (72). Here we investigated the potential role of bacterial surface properties and morphology on the textural and structural parameters of fermented milk. Comparisons were made between engineered strains showing alterations in cell chaining, clumping, exopolysaccharide production, and pili formation and their isogenic parental strains.

We found that the alterations of cell surface morphology are accompanied by changes in surface charge and hydrophobicity. The bacterial surface properties of these strains also affected the viscosity and gel hardness of milk fermented with them. In addition, the bacterial localization in the fermented milk matrix (Table 4) was dependent on cell surface morphology.

Table 4. Summary of textural parameters of milk fermented with surface-altered lactococci.

Phenotype	Strain[$]	Viscosity, %	Gel hardness, %
Pili overexpression	MG1363(pIL253*pil*)	+49.3 **	+19.2 **
	MG1363(pIL253*pil*Δ1)	+45.3 *	+12.5 *
	MG1363(pNZ521;	+40.1 **	+14.7 *
	MG1299(pIL253*pil*)	+ 9.8	-1
	NCDO712(pIL253*pil*)	-20.4 *	-2.5
	IL1403(pIL253*pil*)	+ 9.5	-1.3
Chaining	IL1403Δ*acmAacmD*	+48.3 *	- 5.6**
	MG1363Δ*acmA*	+11.7 *	-5.2*
Clumping	MG1614_clu$^-$	+6.7	+4.3

* - significance ($p<0.05$); ** - significance ($p<0.01$)

[$] All comparisons are made with the isogenic parent strain

The bacterial cell surface contains charged hydrophilic as well as hydrophobic areas, which are the result of the complex molecular composition of the cell wall. For example, overexpression of pili in MG1363(pIL253*pil*) led, in neutral pH, to a decrease in charge (from -26±2 mV to -12±0 mV). The decrease in net-negative charge can possibly be explained by the slightly net-positive charge of the overexpressed pilin proteins. The charged residues of pilin proteins are likely to be on the outside of the pilus (73). Analysis of the *spa* pilin protein sequences (http://www.expasy.org/) showed 30-49% hydrophobic residues. The increase in hydrophobicity from 5% to 96% of MG1363(pIL253*pil*) relative to its parent might be explained by hydrophobic patches on the overexpressed pili. Taken together bacterial amphiphilic surface properties are governed by the molecular composition of its cell wall, which is strongly strain dependent (34), (Chapter 3) and can be engineered.

The increased viscosity and gel hardness of fermented milk using pili overexpression strains was seen independently for three out of the six strains tested, which indicates that this effect is strain-dependent. The three strains where this effect was not observed are NCDO712, MG1299 and IL1403. Strain NCDO712 carries the *spa* pilin genes on the low copy-number plasmid pSH74, which are not expressed at high levels (35), (see also Chapter 2). Similarly, electron microscopy revealed that IL1403(pIL253*pil*) carried low numbers of pili on the surface (35), (Chapter 2). Strain MG1299(pIL253*pil*) forming long chains shows a trend towards higher viscosity but it has no effect on gel hardness. This phenomenon is in agreement with our observations for solely chaining strains described below. The 20% - 35% increase in viscosity obtained with the other strains is a substantial alteration that is likely to be perceived sensorically.

The effect of the microbial surface on textural properties of fermented milk leads us to hypothesize that bacteria can be seen and used as structural or ingredient elements of a food matrix. As such they engage in physico-chemical interactions with milk proteins and/or with other cells via molecules on their polymeric cell surface layers (27–29). The occurrence of interactions is a balance between attractive and repulsive forces, which strongly depend on the interacting particles. For instance, it was shown that for *Lactobacillus bulgaricus* GG the force between micellar casein (or denatured whey protein) and the bacterial cell is about 0.4 nN, but this was significantly different for other strains (25). Together with our results, these observations suggest that the type and force of microbe-matrix interactions can influence the structure, stability, and textural properties of the fermented milk matrix.

Strains of *L. lactis* show a high degree of biodiversity in surface properties among which their capacity to bind to milk proteins. We have previously reported that dairy isolates display a higher milk protein binding affinity than plant isolates (34), (Chapter 3). The increased viscosity and gel hardness of fermented milk might, thus, also be caused by bacterial interactions with milk proteins (34), (Chapter 3) as well as by cell to cell connections via pili between the starter culture cells when they are in a close proximity (35), (see also Chapter 2). The results of the current study show that despite the surface alterations inflicted, the lactococcal cells retained a high capacity to bind to milk

proteins (Table 2). These results indicate that factors other than pili or morphology changes are responsible for milk protein binding.

Interactions of *L. lactis* with milk proteins might result in preferential localization of the bacterial cells in the protein matrix or in serum regions, in the form of aggregates and/or chains. This is corroborated by microscopic observations of pili overexpressing cells in the milk matrix. These cells seem to be localized in the serum regions (Fig. 1). Here the size and shape of particles is of importance. Cells do not only form very strong cell-cell connections, leading to cell aggregates located in milk gel cavities, but also cell-protein contacts between pili of outer cells of aggregates and proteins of milk gel, which appeared to contribute to the increased milk gel hardness.

Similar to pili expressing cells, which show a chaining and, after centrifugation, a clumping phenotype, bacteria that only form chains (*acmA*/*acmD* deletions) increased the viscosity up to 12-48%, and in contrast to pili expressing cells this lead to a decrease in gel hardness by 6-14% (Tables 2 and 3). It was detected that there are more cavities in milk gel fermented with chaining strains. The decreased gel hardness can be explained by cavities of serum in the milk matrix: they do not provide any additional bonds to strengthen the gel. We speculate that that the cells do not interact with the aggregated casein micelles in this case and are, thus, not included in the matrix but act like structure breakers or inert fillers. This would be similar to what is seen for fat droplets with natural milk fat globular membranes in milk gels made with un-homogenized milk (71): in this case, the matrix has to form around those droplets. Also longer cell chains can be considered as "viscosifying" molecules and not as enhancing properties for the gel hardness. Chain length might be at the basis of the increased viscosity of a matrix obtained by fermenting milk with chaining cells. In general, the longer the molecule (especially when it is stretched) the more viscosifying properties it has due to possible hydrogen bonding with water molecules as well as molecule - molecule interactions or entanglements.

The findings presented here show that engineering surface properties of dairy starter strains allows altering product properties such as gel hardness and milk viscosity. It will

be highly interesting to see whether the quality changes can be perceived in sensory analyses. This opens possibilities to develop new concepts in improving fermented products with altered textural properties.

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Competing interests

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Chapter 5

Influence of lactococcal surface properties on cell retention and distribution in cheese curd

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Abstract

During cheese manufacturing, on average 90% of the starter culture cells are believed to be entrapped in the curd, with the remainder lost in whey. This paper shows that plasmid-cured dairy strains of *Lactococcus lactis* show cell retention in the curd of 30%-72%, whereas overexpression of pili on the lactococcal cell surface can increase cell retention to 99%. Exopolysaccharide production and cell clumping and chaining do not influence cell retention in cheese curd. *L. lactis* surface alteration also strongly affected the distribution of cells in the cheese matrix: clumping and overexpression of pili led to formation of large cell aggregates embedded in the protein matrix whereas exopolysaccharide expression resulted in cells being surrounding by small serum regions in the protein matrix of the cheese. These results suggest that surface properties of dairy starter cultures strongly determine retention and distribution of the bacteria in cheese curd.

Introduction

Approximately 90% of the starter culture cells are typically entrapped in the curd during cheese production, whereas the remaining 10% of cells are "lost" in the whey (1, 2). Once entrapped in cheese, *L. lactis* cells keep on growing to colonies of various sizes, which is determining for the pH, flavour profile (3–5), taste and texture (6) of the final product. The spatial distribution and specific localization of bacterial colonies in the curd is suggested to be important for cheese ripening. For instance, it was demonstrated that inoculation density influences the size and spatial distribution of colonies in the cheese matrix (2). The spatial distribution of cells was shown to influence metabolite production during cheese ripening: in cheeses with roughly the same final cell density, higher amounts of metabolites were detected in cheeses with small bacterial colonies than in cheeses with big colonies (7). This phenomenon was related to the differences in surface exchange between the cheese matrix and bacterial colonies (7). It was also reported that in cheese with a higher fat content, bacteria tend to locate increasingly in the vicinity of fat droplets or near the fat-protein interphase (8). In the same study higher cell retention was found in full-fat cheese which was accompanied with increased flavour formation.

Bacterial retention and distribution in the cheese matrix are likely determined by interactions between microbial surface properties, such as surface charge and hydrophobicity, and the properties of e.g. milk proteins and fat droplets. Surface properties of bacteria are determined by the molecular constituents of their cell walls, such as (lipo-)teichoic acids, proteins, pili or capsular polysaccharides (9–11). For example, the presence of pili on lactococcal cell surfaces leads to cell chaining and cell clumping as well as to an increased hydrophobicity and lower negative charge of the cell surface (12) (Chapter 2). Furthermore, milk protein binding seems to be more pronounced in lactococcal dairy isolates compared to plant isolates (13), (Chapter 3). The composition of the bacterial cell wall can affect the textural properties of fermented dairy products through cell chaining, clumping, formation of pili (14), (Chapter 4) or of EPS (15–18). *Lb. rhamnosus* EPS-milk protein interactions, for instance, lead to increased water retention and a softer cheese matrix, increased yoghurt

viscosity and longer texture (16). Incidental evidence showed that a chaining cells of *L. lactis* were in contact with fat droplets and it was suggested that cell chains even could form bridges between fat droplets (18).

Here the impact of *L. lactis* cell morphology, resulting from alterations of surface properties such as decoration with pili, cell chaining and/or cell clumping, on the retention of cells in Gouda-type cheese and on their distribution patterns in the cheese matrix was studied. To this end 10 isogenic *L. lactis* strains were used that only differed in known cell surface properties and/or morphology. The results demonstrate that the presence of pili on the bacterial cell surface significantly increases immobilization of cells in the cheese curd. Furthermore, cell clumping and overexpression of pili lead to the formation of large cell aggregates in the cheese matrix.

Materials and Methods

Bacterial strains, growth and enumeration

The strains used as starter cultures for the manufacturing of Gouda-type cheese are listed in Table 1. They were pre-cultured overnight at 30°C in 20 mL sterilized (115°C, 10 min) full-fat bovine milk (3.5 % fat, 3.3 % protein). For lactose- (Lac$^-$) and protease-negative strains, glucose (50% stock solution dissolved in sterilized full-fat milk) and Bacto™ casitone (BD Biosciences, Breda, The Netherlands) (20% stock solution in demineralized water) were added to final concentrations of 1% and 0.2%, respectively. When required, erythromycin (Ery; 10 μg mL^{-1}), chloramphenicol (Cm; 5 μg mL^{-1}), rifampicin (Rif; 50 μg mL^{-1}), or streptomycin (Str; 100 μg mL^{-1}) were added to the indicated final concentrations.

Table 1. List of strains used for Gouda-type cheese manufacturing in this study.

No	*L. lactis* strains	Characteristic	Reference
1	NCDO712	*L. lactis* ssp. *cremoris* wild type dairy isolate. Lac^+; Contains 6 plasmids - pLP712, pSH71, pSH72, pSH73, pSH74, pMT712	(12, 19)
2	MG1363	Plasmid-cured derivative of *L. lactis* NCDO712; Lac^-	(19)
3	MG1363(pIL253*pil*)	*L .lactis* MG1363 harboring pIL253*pil* encoding the pilin operon *spaCB-spaA-srtC1-srtC2* from NCDO712; Ery^R; Lac^-	(12)
4	MG1363(pNZ4120)	*L. lactis* MG1363 harboring EPS gene cluster from *L. lactis* subsp. *cremoris* B40 on pNZ4120; Ery^R; Lac^-	(20)
5	MG1614	*L. lactis* MG1363; Str^R; Rif^R; Lac^-	(19)
6	MG1614_clu$^+$	*L. lactis* MG1614 transconjugant harboring pLP712 and showing a clumping phenotype. Str^R; Rif^R; Lac^+	(14)
7	MG1614_clu$^-$	*L. lactis* MG1614 transconjugant carrying pLP712 and showing a non-clumping phenotype. Lac^+	(14)
8	IL1403	Plasmid-free derivative of *L. lactis* ssp. *lactis* IL594; Lac^+	(21)
9	IL1403Δ*acmAacmD*	IL1403 in which the *acmA* and *acmD* genes were removed by double cross-over recombination, resulting in a chaining phenotype; Lac^-	(22)
10	IL1403(pIL253*pil*)	IL1403 harboring the pilin operon *spaCB-spaA-srtC1-srtC2* from strain NCDO712 on pIL253; shows chaining phenotype and high hydrophobicity; Ery^R; Lac^-	(12)

Gouda-type cheese production

Gouda-type cheese was made from pasteurized (72°C for 15 s) non-homogenized full-fat bovine milk (3.5% fat, 3.3% protein, 4.5% lactose) supplied by the NIZO pilot plant. To ensure growth of all strains, the milk was supplemented with 0.2% casitone, and with 4% glucose for lactose-deficient strains. The milk (2.0 L) was inoculated with 20 mL of an overnight culture ($\approx 10^9$ CFU mL^{-1}) of the strains listed in Table 1. In contrast to the preculture, for the actual cheese making no antibiotics were added.

Further, 400 μL L^{-1} of a 35% $CaCl_2$ solution and 230 μL L^{-1} rennet (Kalase 150 IMCU mL^{-1}, CSK Food Enrichment, Ede, The Netherlands) were added and the milk was allowed to coagulate for 45 min at 30.5°C. Subsequently, the curd was cut into cubes with an edge length of 0.3-0.5 cm by slowly moving a cutting device through the curds for 10 min. When the curd volume had been reduced to ≈30% of the initial milk volume, and 70% of the original volume had thus been released as whey, an amount of whey equivalent to 40% of the total volume (the 1st whey) was removed and replaced with an equivalent volume of sterile tap water of 45°C. This washing step is typical for Gouda cheese manufacture, with the purpose to wash out lactose (and thus limit final acidity) and to raise the temperature to increase syneresis. The curd was then stirred for 1 min with 10 min intervals during 1.5 h at 36°C. The curd was transferred into cheese moulds and pressed at 27 g cm^{-2} for 1.5 h at room temperature. After 45 min, the cheese was turned upside down in the mould and pressed for another 45 min. The cheese was subsequently incubated for 18 h at 30°C, after which the pH was measured and it was salted for 1 h in brine solution containing 23% salt. After brining, the cheese was dried in a sterile flow cabinet for 1 h at room temperature and vacuum packed under 1% nitrogen, and ripened at 13°C for 12 wk.

Distribution of starter cells between curd and whey

Colony forming units in curd and the 1st whey were determined at the point of removal of the 1st whey, as outlined in section above. For the determination of cell counts, 3 g of curd were mixed with 27 mL of sterile 2% sodium citrate (t = 40°C) and homogenized for 8 min in a Stomacher (Model no. BA 60201, Seward Medical UAC House, London, UK). For each sample of either the 1st whey or the curd, three replicate serial dilutions were prepared and subsequently plated on M17 (Oxoid Ltd, Basingstroke, UK) agar medium containing 1% glucose. Colony forming units (CFU) were quantified after 48 h of incubation at 30°C. The cell retention in curd, taking into account the amount of curd and whey at the time of sampling, was calculated as the fraction of cells in curd as given in Eq. 1. Alternatively the volume corrected fraction of cells retained in the 30% curd was calculated as outlined in Eq. 2. This correction gives a clearer picture

of cell retention in curd as the final curd/whey ratio changes throughout the process and this calculation gives a volume independent measure. Throughout the chapter - the volume corrected fractions were used for comparisons.

Equation 1:

$$Cell\ fraction\ in\ curd = 100 \times \frac{CFU\ per\ g\ curd}{(CFU\ per\ g\ curd + CFU\ per\ mL\ whey)}$$

Equation 2:

$$Volume\ corrected\ cell\ fraction\ in\ curd\ = 100 \times \frac{30 \times CFU\ per\ g\ curd}{(30 \times CFU\ per\ g\ curd + 70 \times CFU\ per\ mL\ whey)}$$

Localisation of cells in the cheese matrix

Confocal laser scanning microscopy (CLSM) was applied on cheese samples after 12 wk of ripening. From the centre of a cheese sample a slice of 2-3 mm thickness and 4-5 mm in length was cut with a sterile scalpel blade. A mixture of 0.5% Acridine Orange (AO) (Sigma-Aldrich, Schnelldorf, Germany) and 0.025% Rhodamine B (Sigma-Aldrich, Schnelldorf, Germany) in water was placed on top of the cheese slice to stain bacteria and the protein matrix, respectively. Surplus dye was removed after 1-2 min, and the specimen was placed on a 25 x 50 mm glass slide such that firm contact was formed between the cheese and the glass slide. Confocal images were taken using a Leica TCS SP 5 confocal laser-scanning microscope (Leica, Mannheim, Germany) with Leica application Suite Advanced Fluorescence software v. 2.7.3. build 9723. The Argon laser was used to visualize the bacteria stained with AO, while the DPSS 561 laser was used to visualize the cheese protein matrix stained by Rhodamine B. Fat droplets remained unstained.

Statistical analysis

Results were analysed using Microsoft Excel. Pairwise comparisons of cell distribution between surface altered and their parental strains were analysed with a two-tailed t-test and considered significant if p-values of were smaller than 0.01.

Results and Discussion

Distribution of cells between curd and whey

To investigate the effect of *L. lactis* surface properties on bacterial cell retention in cheese curd and the patterns of cell localisation in the cheese matrix, 10 strains of surface-engineered isogenic *L. lactis* strains were used. The surface morphology of the strains was modified in terms of cell chaining, clumping, EPS formation and pili expression. EPS formation was achieved by introducing the EPS gene cluster from *L. lactis* subsp. *cremoris* B40 (20) into strain MG1363. The production of pili was achieved through expression of the Spa-pilin gene cluster *spaCB-spaA-srtC1-srtC2*, from plasmid pSH74 of strain NCDO712, and cloned in the multi-copy plasmid pIL253 (12), (Chapter 2). To our knowledge, this is the first study where surface altered lactococci were used to study cell retention in curd.

The results showed that 89% of the starter culture was retained in the curds when the wild-type *L. lactis* subsp. *cremoris* dairy isolate NCDO712 was used, but this decreased to 30% for its plasmid-free derivative MG1363. The plasmid-cured *L. lactis* subsp. *lactis* strain IL1403 showed 53% cell retention. Overexpression of the EPS cluster in MG1363(pNZ4120) also resulted in a low retention of cells in the curd (33.7±4.6 %). The results of cell retention in curd for the wild type strain NCDO712 are consistent with literature findings (1). However, cell retention of plasmid cured NCDO712 and IL594 derivatives MG1363, MG1363(pNZ4120) and IL1403 are much lower. The plasmid cured strain MG1614 was selected for spontaneous resistance to rifampicin and streptomycin and shows significantly increased cell retention in curd compared to its parent strain MG1363. While this increased retention in cured coincides with

decreased zeta potential and increased hydrophobicity (14), (Chapter 4) the molecular link to this phenotype is not clear.

The loss of cells in the whey fraction could arise from several processes. It could be caused by release of cells from the surface of curd granules after cutting of the curd, similar to the loss of some of the fat globules to whey (23). However, assuming an even cell distribution and the loss of all cells in the outer 10 μm surface layer of a curd particle with the dimensions of 5x5x5 mm, this would account for <1% of cell loss. Additionally, higher losses could possibly take place if large clusters of cells are present in weak conglomerates in the matrix at the position where cutting of curd occurs. However, even those losses are unlikely to explain the loss of 10% or more of cells into the whey. Hence, there appears to be a mechanism of 'active removal' of cells from the cheese curd. This is most likely related to the syneresis process where whey is drained from the curd and any materials unattached to the matrix can be removed with the whey if their size is smaller than the pores in the curd matrix.

After rennet-induced hydrolysis of κ-casein in milk, the volume fraction of para-casein micelles is ≈8-10%, with fat globules and bacterial cells suspended in the serum phase. At this point, the pores are sufficiently large for bacterial cells and fat globules to be removed with the whey (24). However, as this pore size gradually decreases during syneresis, the expulsion of cells is ultimately hindered, leading to their entrapment as well as that of fat globules in the curd, as previously described by Laloy et al. (1996).

Table 2. Distribution (%) of cells between whey and curd. For comparisons surface altered strains are grouped with their isogenic parent. Significance levels (* indicates p<0.01) are in comparison with the parental strain.

Strain	CFU/mL whey		CFU/g curd		Cell fraction in curd (%)[$]		Volume corrected cell fraction in curd (%)[$]	
	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD
Wild type dairy isolate								
NCDO712	1.73E+06	1.29E+06	1.94E+08	1.86E+08	94.6	5.7	89	11.7
Pili overexpression								
IL1403	4.23E+06	3.46E+06	1.03E+07	6.66E+06	65.4	31.2	53.4	36.4
IL1403(pIL253*pil*)	2.72E+05	1.17E+04	5.98E+07	9.28E+06	99.5*	0.1	98.9*	0.2
MG1363	1.54E+07	1.28E+07	1.42E+07	1.13E+07	49.7	8.2	30.2	7.7
MG1363(pIL253*pil*) (colony 1)	6.62E+05	3.48E+05	1.73E+07	1.18E+07	95.7*	1.3	90.6*	2.6
MG1363(pIL253*pil*) (colony 2)	1.76E+06	1.43E+06	8.08E+07	7.29E+07	97.4*	0.8	94.2*	1.8
Mixture of EPS and pili producing strains								
MG1363(pNZ4120)	5.62E+06	1.18E+06	6.77E+06	1.99E+06	54.1	5.2	33.7	4.6
MG1363(pIL253*pil*) + MG1363(pNZ4120)	5.23E+05	3.73E+05	4.48E+07	3.45E+07	98.8*	0.3	97.1*	0.7
Clumping phenotype								
MG1614	6.56E+06	2.18E+06	3.83E+07	6.22E+06	85.1	5.5	71.5	8.7
MG1614_clu$^-$	7.40E+06	1.21E+06	6.35E+07	1.70E+07	89.0	3.4	77.8	6
MG1614_clu$^+$	4.32E+06	1.21E+06	2.23E+07	3.03E+06	83.7	4.8	69	7.6
Chaining phenotype								
IL1403	4.23E+06	3.46E+06	1.03E+07	6.66E+06	65.4	31.2	53.4	36.4
IL1403Δ*acmAacmD*	7.60E+06	6.78E+06	1.13E+07	1.16E+06	64.5	17.2	45.6	17.4

[$] Fractions of cell retention were calculated per sample and subsequently the mean and SD were determined.

Taken together, it appears that bacterial cell surface properties can affect retention of the cells in the cheese curd. This could either be due to interactions of the bacteria with the matrix, or to cell clustering, which would lead to an increased size and more restricted movement through the curd matrix. Clumping of bacteria, however, did not significantly affect cell retention in the cheese curd (Table 2). Interestingly, overexpression of the Spa-pilus gene cluster in strains MG1363 and IL1403 increased cell retention in the curd, to about 90.6-98.9% (Table 2). The co-culturing of the EPS-expressing MG1363(pNZ4120) with Spa-pili overexpressing MG1363(pIL253*pil*) increases cell retention in the curd to up to 97.1±0.7%.

The fact that the expression of pili in only a part of the starter culture cells in the curd is sufficient for cell retention suggests that interactions take also place between the pili producers and non-producers. Pili overexpression also alters properties such as cell surface hydrophobicity and zeta potential (14), (Chapter 4). To distinguish between the direct effects of pili and putative other surface properties on cell retention in curd, two derivatives of MG1363 were used, in which the lactose/protease plasmid pLP712 had been transferred via conjugation (14), (Chapter 4). One of these transconjugants, MG1614_clu^{+}, has a clumping phenotype and a significantly higher surface hydrophobicity and slightly lower net-negative charge than its parent, MG1363. The other transconjugant, MG1614_clu^{-}, does not clump and has surface properties similar to those of MG1363, except for hydrophobicity, which remained ≈70-80% (14), (Chapter 4). Using these strains for cheese-making showed that surface alterations except for pili overexpression did not affect cellular distribution in curd and whey.

Together, this data indicates that overexpression of Spa-pili in different lactococcal strains consistently leads to the retention of a higher fraction of cells in cheese curd while cell hydrophobicity and zeta potential alterations, due to pLP712 plasmid transfer into MG1363 did not have such effects. Cell retention is significantly lowered as a consequence of curing of the six plasmids from strain NCDO712 (compare the results of NCDO712 with those of its plasmid-free derivative MG1363). The plasmids of NCDO712 code for several dairy-related properties such as lactose utilization, an extracellular protease, an endopeptidase, peptide transport and others (12, 25). Plasmid

pSH74 of NCDO712 encodes the pilin gene cluster *spaCB-spaA-srtC1-srtC2* that was overexpressed in several of the strains used in this study. The relatively high cell curd-retention reported here for strain NCDO712 and previously for other dairy strains suggests that this might be a property of starter strains that has been selected for.

Distribution of cells with altered surface properties in the cheese matrix

To investigate whether bacterial cell surface alterations can affect the distribution of cells in the cheese matrix, 12 week-old cheese samples were examined by CLSM imaging. All strains described above were used in these examinations. Three main phenomena with respect to bacterial distribution in the cheese matrix were observed: 1) small groups of cocci are randomly embedded throughout the matrix, 2) cells are present as aggregates, 3) EPS producing cells seem to be surrounded by small serum regions of the protein network.

The first of these distribution patterns was seen for the plasmid-free strains MG1363 and IL1403. The cells of these strains were predominantly present as small groups of cocci entrapped throughout the protein matrix of the cheese (Fig. 1A and E). The second apparent distribution pattern was in form of cell aggregates that occurred upon overexpression of the pilin operon *spaCB-spaA-srtC1-srtC2* in MG1363 (Fig. 1B). A similar trend was seen for Spa-pili overexpressing *L. lactis* IL1403 (data not shown). Interestingly, the chaining phenotype observed in IL1403Δ*acmAacmD* and the clumping transconjugant *L. lactis* MG1614_clu^{+} also led to formation of cell aggregates in cheese (Fig. 1F and 1D). The third distribution pattern was seen for the EPS-producing MG1363(pNZ4120): cells of this strain seem to be surrounded by small serum regions (Fig. 1, C). Overall, the CLSM results indicate that alterations in cell chaining, clumping, EPS production or Spa-pili overexpression influence the distribution of lactococcal cells in the cheese matrix.

Based on the described findings we propose that cells with altered surface properties may have altered functionalities in cheese. This is in line with an earlier study showing that the alteration of cell surface morphology (chaining, clumping, EPS formation, pili expression) not only affects cell surface charge, hydrophobicity and the attachment of cells to proteins, but it can also lead to differences in gel hardness and viscosity of milk fermented with the engineered strains (14), (Chapter 4). The current study indicates that by altering surface properties of dairy starter cultures it is possible to minimize the loss of cells in whey during cheese manufacturing, which might be applied to create a cleaner whey or to alter textural and, ultimately also, sensory qualities of cheese.

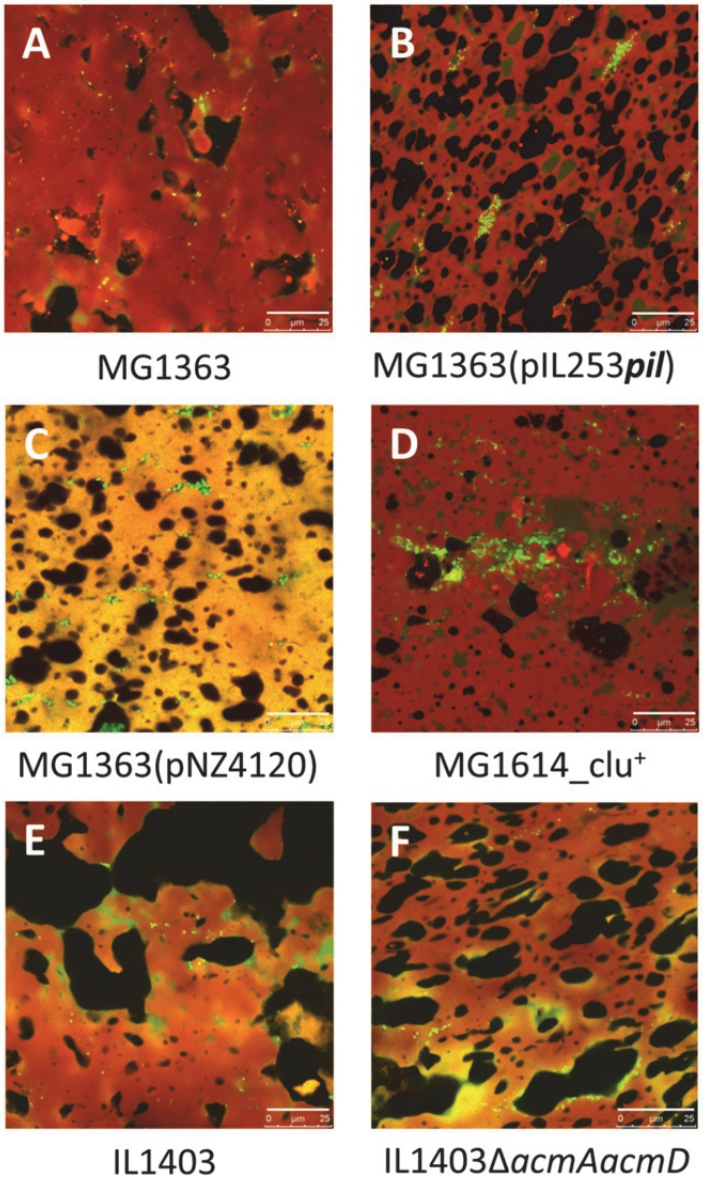

Figure 1. Microstructure of Gouda-type cheese made with *L. lactis* strains with altered surface properties. Bacterial cells are green, the protein matrix appears orange/red; the black areas represent oil droplets, air pockets or serum regions. White bars: 25 µm. (A) *L. lactis* MG1363, (B) *L. lactis* MG1363(pIL253*pil*) overexpressing Spa pili and leading to a chaining, clumping and hydrophobic phenotype, (C) EPS-producing *L. lactis* MG1363(pNZ4120), (D) clumping and hydrophobic transconjugant MG1614_clu^{+}, (E) *L. lactis* IL1403, and (F) *L. lactis* IL1403Δ*acmAacmD*, exhibiting a chaining phenotype.

Conclusions

L. lactis cell surface properties play an important role in the distribution and retention of starter culture cells in curd during cheese making. While the curing of plasmids from a wild type dairy isolate, *L. lactis* NCDO712, leads to a decrease in cell retention, overexpression of the pilus gene cluster *spaCB-spaA-srtC1-srtC2* results in a significant increase in cell-retention in the model strains of the two *L. lactis* subsp. *cremoris* MG1363 and *L. lactis* subsp. *lactis* IL1403. The alteration of cell retention in curd might open possibilities to modify starter culture functionality as well as whey and cheese quality.

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Chapter 6

Bacterial emulsification by and transcriptional response of *Lactococcus lactis* residing at an oil-water interphase

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Abstract

Microbial surface properties are important for interactions with the environment in which cells reside. Surface properties of lactic acid bacteria significantly vary and some strains can form strong emulsions when mixed with a hydrocarbon. Here, we show that *Lactococcus lactis* NCDO712 forms oil-in-water emulsions when mixing a cell suspension with petroleum. In the emulsion the bacteria locate at the oil-water interphase which is consistent with Pickering stabilization. Cells of strain NCDO712 mixed with sunflower seed oil did not stabilize the oil droplets. Addition of ethanol or ammonium sulfate led to cell aggregation, which subsequently allowed stabilizing such emulsions. From this, we conclude that bacterial cell aggregation is important for emulsion droplet stabilization. To determine how such locally very high cell concentrations at the oil-water interphase influence the microbial transcriptome, RNAseq analysis was performed on the RNA isolated from lactococci residing at the oil-water interphase. In comparison to cells in suspension 72 genes were significantly differentially expressed with a more than 4-fold difference. The majority of these genes encode proteins involved in transport processes and the metabolism of amino acids, carbohydrates and ions. Our results are relevant for considering lactic acid bacteria as clean label emulsion stabilizers. In addition, microdroplet technologies are increasingly used in research. The understanding of interactions between bacterial cells and oil-water interphases is of importance for conducting and interpreting such experiments.

Introduction

The interactions between microbial cells and substrates or solid surfaces can be attractive or repulsive and depend on properties such as temperature, pH, ionic strength, roughness of a surface, hydrophobicity or surface charges (1, 2). Bacterial adhesion has been studied in relation to bacterial infections (3), adhesion to environmental systems, e.g., intertidal systems with subsequent biofilm formation (4–6), biomedical applications (7), as well as bioremediation and fermentation processes (8–10). Gram-positive bacteria have been shown to function as emulsifiers of hydrocarbons e.g., petroleum, without involvement of cell growth and substrate degradation (11–13). Such emulsification is caused by microbial cells locating on the oil-water interphase, which prevents droplet coalescence and leads to so called Pickering-stabilization of emulsions (14, 15). The droplet size distribution of emulsions stabilized by microbial cells is in the range between 100-500 μm (16); the stabilizing particle size should be at least an order of magnitude smaller than the emulsion droplet size (17). Bacterial cells are often simplified to solid particles in order to describe such emulsions (18, 19). The contacts between these solid particles and the surfaces of emulsion droplets are typically explained by van der Waals and electrostatic interactions and they are united in the so-called DLVO theory (named after Derjaguin, Landau, Verwey, and Overbeek) (20). Most bacterial surfaces are negatively charged and can be regarded as charged colloidal particles in aqueous systems (16, 19). Bacterial cell wall molecules such as proteins or polysaccharides will attract counter ions from the surrounding environment and, together, form an electrical double layer around the cell (21). Thus, the pH and ion concentration of the surrounding environment has been suggested to affect the location of bacteria at the oil-water interphase of an emulsion (22). However, a generic explanation for microbe-matrix adhesion interactions was not obtained by considering bacteria as charged colloidal particles with a surrounding electric double layer. The addition of short-range Lewis acid-base interactions or hydration, and steric interactions led to the extended XDLVO theory (19, 23). However, even XDLVO does not fully explain microbe-matrix interactions, probably because of the high cell surface complexity, which significantly differs between bacteria and non-biological particles (18,

24). Indeed, the molecular composition of Gram-positive bacterial cell surfaces is quite diverse (25, 26), providing cells with different surface properties (27–29). The resulting differences in e.g. charge (25, 30) and hydrophobicity (31, 32) are involved in bacterial interactions with interphases (2, 33). By contrast, the surface of solid spherical particles is uniformly charged or hydrophobic. Adding to the complexity is the fact that interactions between bacteria and substrates can be strain-specific (15, 34).

Examples of undesirable bacterial emulsification can be found in biofuel production where oil-producing bacteria can stabilize biofuel oil droplets as Pickering stabilization particles in water, which further impedes biofuel recovery (35). The number of bacterial species that have been described to facilitate Pickering stabilization is still limited (12, 16, 36). It was recently reported that lactic acid bacteria (LAB) can be applied as solid particles for the production of food-grade Pickering emulsions (16, 37). The influence of LAB surface properties on food emulsions already stabilized by a surfactant was investigated and the results suggest that they play an important role in interactions of bacteria with emulsion matrix components (38, 39). We recently also showed that by altering the surface of the LAB *Lactococcus lactis* through e.g. the overexpression of lactococcal pili, the gel hardness and the viscosity of a fermented milk product made with this organism were changed (40), (Chapter 4). While there is a reasonable amount of knowledge on how bacteria in food fermentations influence textural properties of the fermented food matrix (10, 41–43), little information is available on molecular mechanism involved in these interactions (27, 41) or on whether and to which extent the location of bacteria on e.g. an oil-water interphase might influence their behaviour.

We hypothesized that altering cell surface properties may allow changing emulsification properties of bacteria. This supposition is based on the fact that chemicals such as acetic and succinic anhydrides, carbodiimide and ethanolamine or ethylenediamine can modify cell surface charge, isoelectric point or water contact angles (44). Calcium ions influence bacterial adhesion to piglet epithelial cells (45), high concentrations of ammonium sulfate cause cell aggregation (46) and even small differences in growth media can change the bacterial cell surface properties (47). Here we evaluate to what

extent cell aggregation caused by ammonium sulfate or ethanol influences the surface hydrophobicity of strains of *L. lactis* and how this impacts the oil emulsification process. To unravel a possible effect of bacterial emulsification on bacterial gene expression, we investigated how residing at an oil-water interphase, as is the case in bacterial Pickering emulsions, influences the transcriptome of the cells.

Materials and Methods

Bacterial strains, growth conditions and medium

Bacterial strains and plasmids are presented in Table S1. *L. lactis* strains were grown at 30°C in M17 (Oxoid, Thermo Scientific, Basingstoke, Hampshire, UK) supplemented with 1% lactose (LM17). When required, rifampicin (Rif; 50 μg/mL), streptomycin (Str; 100 μg/mL) or erythromycin (Ery; 10 μg/mL) was added to the indicated end-concentrations.

Aggregation measurements

Cell from overnight cultures were harvested by centrifuging at 6037 g for 3 min in 50 mL tubes, washed twice with phosphate buffer (10 mM, pH 6.8), and finally diluted to an optical density at 600 nm (OD_{600}) of 1.0 in the same buffer.

The cell suspensions (1.5 mL each) were transferred to 2 mL Eppendorf tubes, centrifuged at 15339 g for 30 sec, after which the supernatant was removed and the cell pellets were re-suspended in phosphate buffer containing 5-25% (v/v) ethanol. Subsequently, the OD_{600} was measured every 10 min for 1 h. The same approach was used to prepare bacterial samples in 10 mM phosphate buffer with 0.1 – 3.0 M ammonium sulfate. Cell aggregation was determined using Eq. 1:

$$Cell\ aggregation\ (\%) = 100 \times \frac{OD_{600}\ at\ 0\ min - OD_{600}\ at\ 1\ h}{OD_{600}\ at\ 0\ min} \qquad [1].$$

Emulsion preparation

Emulsions were prepared as described previously (29), (Chapter 3). The oil used was either petroleum (Sigma-Aldrich, Steinheim, Germany) or plant-derived oil (sunflower seed oil from *Helianthus annuus* (Sigma-Aldrich, #S5007-1L, Steinheim, Germany). For experiments with ethanol or ammonium sulfate either 5-25% ethanol or 0.1-3.0 M ammonium sulfate was added to the cell suspension prior to mixing with the oil. Five mL of the cell suspension in 10 mM phosphate buffer (with or without ethanol or ammonium sulfate) were mixed with 2 mL of the various oils including petroleum. The mixture was vortexed for 2 min and allowed to stand for 15 min for phase separation prior the measurements of OD at 600 nm.

Confocal Laser Scanning Microsopy (CLSM)

Prior to the measurement cells from an overnight culture (10 mL of OD_{600} = 1) were spun down, washed twice with 10 mM phosphate buffer pH 6.8, and re-suspended in 100 µL of the same buffer. At room temperature and protected from light the cells were stained for 30 min, with Syto 60 (Thermo Fisher Scientific, Oregon, Hillsboro, USA) by adding 1 µL of the staining solution (5 mM in DMSO) to 1 mL of cell suspension (OD_{600}=1). Buffer (10 mM phosphate) was separately prepared by adding carboxyfluorescein (200 mL buffer + 300 µL of 100 mM carboxyfluorescein (Sigma-Aldrich) stock solution in water) and kept out of the light.

After staining with Syto 60, the cell suspension was diluted till OD_{600} of 1 in the carboxyfluorescein buffer. This suspension (5 mL) was mixed with 2 mL petroleum, vortexed and allowed to stand for 20 min in the dark for proper phase separation. Then 300 µL of the emulsion (top layer) was transferred into a CLSM cuvette (NIZO, Ede, The Netherlands).

CLSM images were acquired with a Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems CMS GmbH, Mannheim, Germany) with Leica application Suite

Advanced Fluorescence v. 2.7.3. build. 9723. The Argon laser with excitation wavelength of 488 nm was used to visualize the carboxyfluorescein-stained buffer phase, while the HeNe633 laser with excitation wavelength of 633 nm was employed to visualize bacterial cells stained with Syto 60. The objective lens used was a Leica HCX PL APO 63×/1.2 /water CORR CS.

Sample preparation for RNA sequencing

An overnight culture (27 mL, OD_{600}=1.58) of *L. lactis* NCDO712 grown in chemically define medium (64) with 1 % lactose (LCDM) was diluted into 800 mL pre-warmed (30°C) fresh LCDM to an OD_{600} of 0.1 and distributed in 25-ml aliquots over 16 tubes of 50 mL. The cultures were incubated at 30°C until an OD_{600} of 0.43±0.03 was reached. Cells were harvested by centrifugation and re-suspended in 25 mL of fresh LCDM to an OD_{600} of 0.4. This cell suspension was mixed with 5 mL of the fluorinated oil HFE7500 (M3) and vortexed for 2 min in a Vortex Genie 2 vortexer (Scientific Industries, VWR International, Darmstadt, Germany) at maximum speed. The resulting oil-in-water emulsion was incubated at 30°C and samples (30 mL each) were taken after 0, 10, 20 and 30 min. As a control, cells were treated as above but the cell suspension did not contain HFE7500. All samples (30 mL each) were prepared in biological duplicates. The suspension and emulsion samples at time 0 min were immediately frozen in liquid nitrogen. Similarly, samples incubated for 10, 20 or 30 min were quick-frozen in liquid nitrogen. To break an emulsion, the sample was centrifuged at 2°C for 3 min at 6037 g. The supernatant was removed and the cell pellet was re-suspended in 400 µL ice-cold Tris-EDTA-(TE)-buffer (pH 8). The cell suspension was transferred to a screw-cap tube containing 500 mg glass-beads (diameter of 75-150 µm). Freshly prepared extraction mixture (500 µL acidic phenol/chloroform (ratio 1:1), 30 µL 10% sodium dodecyl sulfate, and 30 µL 3M Na-acetate (pH 5.2)) was then added and the tube was frozen in liquid nitrogen and kept at -80°C before breaking the cells. Cells were broken in a Savant FastPrep FP120 "bead beater" (Thermo Savant, Illkirch, France) by beating three times for 40 s at a speed of 4.0

m/sec. The sample was cooled for 1 min on ice in between the steps. Subsequently, the suspension was centrifuged for 1 min at 4°C at 14000 g in an Eppendorf centrifuge (Marshall Scientific, Hampton, NH, US). The supernatant (500 µL) was transferred to a fresh eppendorf tube, mixed with 400 µL cold (4°C) chloroform, and centrifuged for 1 min in an Eppendorf centrifuge at 14000 g at 4°C to improve RNA yield. RNA was isolated with the High Pure RNA Isolation Kit (Roche Molecular Systems, Almere, The Netherlands) using the protocol of the manufacturer. RNA concentration was determined using a Nanodrop (Thermo Fisher Scientific, Wilmington, DE, US). RNA samples were sent for nucleotide sequencing (PrimBio Research Institute, Exton, USA) using an Ion Proton system using an Ion P1-chip (Life Technologies).

Data analysis

Each sample had on average 9.1 ± 1.4 million reads. Raw gene expression data for the two biological replicates per sample were normalized for total counts per sample and analysed using EdgeR (65) with multiple testing corrected *p*-value using the false-discovery rate (method used: Benjamini & Hochberg). Genes with a *p*-value below 0.01 and differential expression levels between emulsion and suspension higher than 4 fold were selected for further visualisation. Data visualization was done using R (https://cran.r-project.org/bin/windows/base/). The d3heatmap function using Euclidian distance matrices, average hierarchical clustering and data scaling was used to generate the heatmap.

Results

Lactococcus lactis can stabilize oil-in-water emulsions

The characterization of *L. lactis* cell surface properties revealed considerable diversity between strains in their propensity to emulsify hydrocarbons (29), (Chapter 3). For further research two strains with the same genetic background but with opposite emulsification properties were selected (Table S1). *L. lactis* NCDO712 cells (99% hydrophobicity) form emulsions when they are mixed with petroleum (Fig. 1B) while cells of *L. lactis* MG1363, a plasmid-free derivative of strain NCDO712, (6% hydrophobicity) do not form such emulsions (Fig. 1A). We have shown earlier that overexpression of the lactococcal pilin gene cluster *pil*, in strain MG1363*pil*, leads to high cell surface hydrophobicity and strong emulsification properties (40), (Chapter 4). To identify the type of emulsion formed by strain NCDO712 we labelled the water phase (buffer) with the green fluorescent dye carboxyfluorescein and the bacterial cells with the DNA stain Syto 60, which fluoresces in the red spectrum. Subsequently, we analysed the emulsion using confocal laser scanning microscopy (CLSM). The images show that a dense layer of bacterial cells surrounds the petroleum droplets while the buffer forms the continuous phase of the emulsion (Fig. 1C). This analysis established that the bacterial cells are located at the oil-water interphase, forming an oil-in-water Pickering emulsion (Fig. 1C).

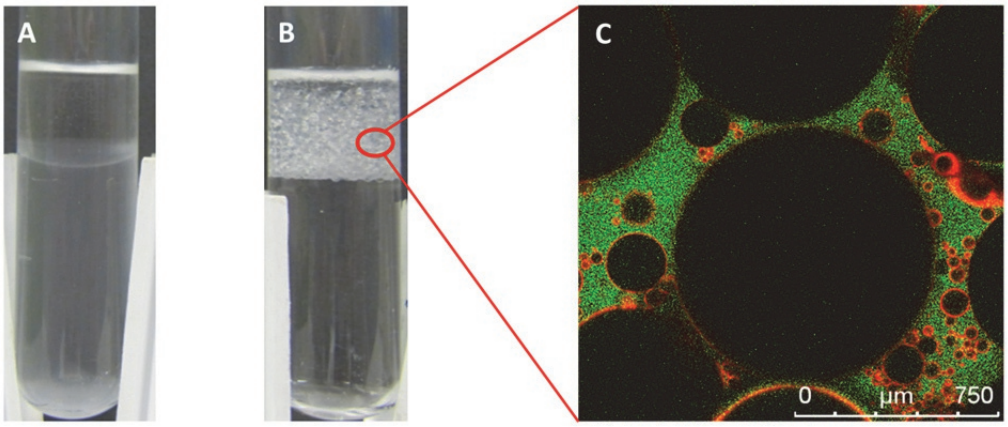

Figure 1. Emulsification of petroleum by *L. lactis*. (A) A suspension of overnight-grown *L. lactis* MG1363 cells in 10 mM phosphate buffer (pH 6.8), after vigorous shaking with petroleum, shows no emulsification of the oil phase (top layer). The cells can be seen in the lower phase (compare with (B)). (B) *L. lactis* NCDO712 produces an emulsion in petroleum with 99% of the cells residing at the oil-water interphase (top layer). (C) CLSM image of the oil-in-water emulsion made with *L. lactis* NCDO712. Petroleum droplets are not fluorescent (black), buffer containing the dye carboxyfluorescein is green (continuous phase) and bacterial cells are red. Due to the polydispersity of the droplets the position in depth differs for individual droplets and therefore different densities of cells are visualized on the oil-water interphase. Size marker is indicated in white.

Cell aggregation influences cell emulsification properties

Pickering emulsification of petroleum was easily done with strain NCDO712 and we wondered if this would also be the case with other types of oil. With sunflower seed oil, which was free from natural emulsifiers, no or only little emulsification was observed (Fig. 2).

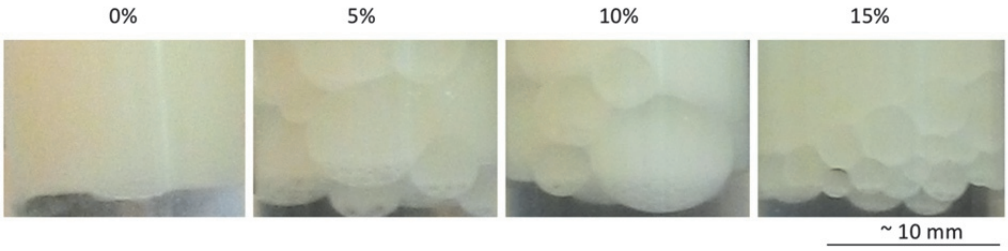

Figure 2. Emulsification as a result of *L. lactis* NCOD712 cell aggregation upon ethanol addition. Addition of ethanol (% (v/v), percentages given above the pictures) to 5 mL of cells from a stationary phase culture of *L. lactis* NCOD712 (OD_{600} = 1), re-suspended in 10 mM phosphate buffer (pH 6.8) allows stabilizing sunflower seed oil emulsion droplets. Ethanol concentrations higher than 20% did not lead to considerable emulsification (not shown). Controls without cells did not lead to emulsion droplet stabilization.

We noted that strains with a clumping/aggregating phenotype are more likely to emulsify petroleum as NCDO712, MG1363*pil* and MG1614_clu$^+$ do (Table S1) than strains that do not aggregate. Based on this observation we hypothesized that cell aggregation might contribute to bacterial emulsification because the energy for stabilization of oil droplets will increase with the radius of the particles formed by the aggregated bacteria (17, 48).

Ammonium sulfate can cause cell aggregation by a mechanism of "salting out" of proteins (46). We also tested whether ethanol could aggregate cells and to what extent this would influence emulsification. Control samples without cells, consisting of buffer with ethanol or ammonium sulfate and oil only, did not result in any emulsion formation. Aggregation of cells in a buffer was observed 1 h after addition of more than 5% ethanol or 0.1 – 3.0 M ammonium sulfate. The addition of 5% ethanol to the cell suspension led to an increase in cell aggregation and the propensity to form emulsions with sunflower seed oil (Fig. 2), (Table 1). The further increase of the ethanol concentration gave variable results (data not shown) which might be due to effects on the cells themself or by direct effects on emulsion formation. The addition of 0.1 M ammonium sulfate led to a clear increase of surface hydrophobicity while concentrations of 2 M or more were needed to see measureable effects on cell aggregation (Table 1). The fact that either the addition of ethanol or ammonium sulfate

resulted in increased cell aggregation and subsequently improved oil emulsification, suggests that cell aggregation aids bacterial emulsification properties.

Table 1. Cell surface hydrophobicity (CSH, %) of *L. lactis* NCDO712 in sunflower seed oil-based emulsions under cell aggregation conditions: ethanol or ammonium sulfate. Results are the average (AV) of 3 replications with standard deviation (STD).

Ethanol			Ammonium sulfate		
Concentration, %	Aggregation, %	CSH, %	Concentration, M	Aggregation, %	CSH, %
	AV±STD	AV±STD		AV±STD	AV±STD
0	12.1 ± 0.1	0.1 ± 0.1	0	8.4 ± 0.9	0.1 ± 0.1
5	52 ± 0.6	74.7 ± 3.5	0.1	1.7 ± 1.5	73.6 ± 0.4
10	54.4 ± 0.9	66 ± 1.8	0.5	5.6 ± 0.6	44.6 ± 0.5
15	31.3 ± 1.1	67 ± 2.5	1.0	2.2 ± 1.2	55.6 ± 0.5
20	32.3 ± 1.7	87.6 ± 0.1	2.0	16.2 ± 1.1	96.2 ± 0.5
25	7 ± 6.1	50.1 ± 1.1	3.0	80.6 ± 4.4	99.7 ± 0.1

Transcriptome response of *L. lactis* cells residing at an oil-water interphase

While there is a reasonable amount of knowledge on how starter culture cells can influence the properties of the matrix of a product during its manufacturing, little is known on possible converse interactions, namely whether the product matrix might influence the starter cells. Here, we studied the Pickering-type of emulsion stabilized by *L. lactis* cells and show that in such emulsions the cells are located in the oil-water interphase. To examine the microbial response to such an environment, we investigated the transcriptome response of *L. lactis* cells to residing at the oil-water interphase.

L. lactis NCDO712 cells were taken either from a suspension or from the oil-water interphase of an emulsion after 0, 10, 20 or 30 min residing time. RNA was subsequently isolated for RNAseq analysis. Emulsions were made with the fluorinated oil HFE7500 (Fig. 3), which is nontoxic as it allows culturing of lactococci in oil-in-water emulsions (49). The majority of cells in such a system are located on the oil-water

interphase, which can be deduced from the fact that an increase in the number of cells added to the system allows to generate larger numbers of smaller oil droplets.

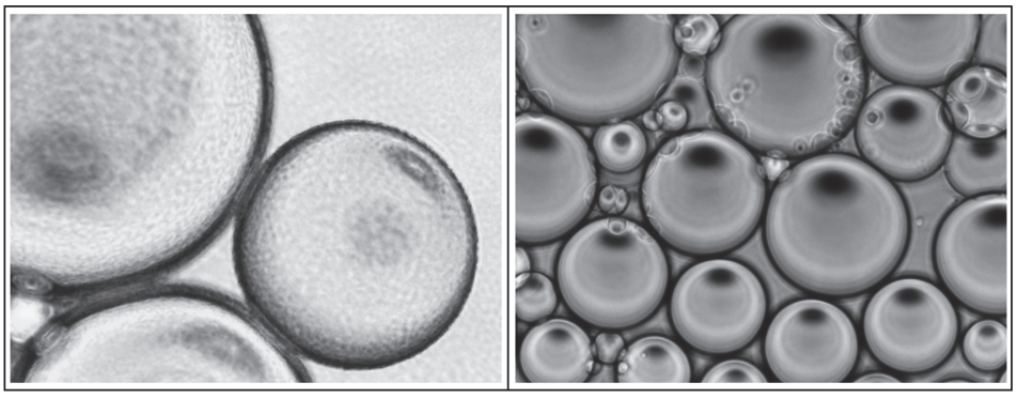

Figure 3. Oil (fluorinated HFE7500)-in-water emulsion stabilized by *L. lactis* NCDO712. Fluorinated oil (2 mL) was mixed by vortexing with 5 mL of cell suspension (OD_{600} = 1) in 10 mM phosphate buffer (pH 6.8). The rough droplet surface is caused by multiple layers of bacterial cells covering the droplets (left panel). The surface is smooth when water droplets are stabilized using the same oil but supplemented with a surfactant (49) (right panel).

First, a clustering analysis of the RNAseq data was performed. Overall, replicate samples cluster together, an indication of good reproducibility of the experiments. Clustering was also observed for either emulsion or suspension samples taken at 0, 10 and 20 min (Fig. S1). The transcriptional response of lactococcal cells to residing at an oil-water interphase differs from that of cells in suspension at the equivalent time point. Interestingly, the transcriptomic response of the cells present for 30 min in emulsion converges to that in the cells kept for 30 min in suspension. Due to the high cell densities required to form a proper emulsion, it is likely that the transcriptional response after 30 min is dominated by acidification and subsequent entering into the stationary growth phase. One of the emulsion samples taken after 20 min of incubation, Emul20.rep1, clusters with the 30 min samples (Fig. S1), indicates that the cells in this sample reached stationary phase somewhat earlier.

For a more detailed analysis of gene expression under the two conditions employed, genes with higher than 4-fold differential expression (p-value < 0.01) were selected. The

analysis was performed for all replicate samples taken after 10 min of incubation of the cells in both conditions because the initial transcriptional response of the lactococcal cells residing on an oil-water interphase differs from that of the cells in the suspension at the equivalent time point (10 min) according to cluster analysis. In total 72 genes were thus analysed, 28 of which are involved in amino acid transport and metabolism (Table 2 and Table S2). Other groups of highly differentially expressed genes encode proteins involved in inorganic ion transport and metabolism (10 genes) and sugar transport and metabolism (6 genes). Another 16 genes have unknown or no predicted functions (Table S2). In contrast to the 10 min samples, the 30 min samples converged, possibly because cells reached stationary phase, which resulted in only 2 genes being differentially expressed: *enoB* (*llmg_pseudo_08*) (7.0 fold change; p=0.0007) and *pSH73_05* encoding a hypothetical protein (-18.4 fold change, p=0.002).

Table 2. Differential gene expression in *L. lactis* NCDO712. Genes presented were more than 4-fold differentially expressed (p-value < 0.01) after 10 min of incubation in an oil-in-water emulsion or in suspension. The genes are classified according to their COG functions (66). Gene clusters according to (61) are marked in bold.

Gene ID [COG function]	Gene Name	Gene annotation	Fold change of Emul10/Susp10	p-value
C. Energy production and conversion				
llmg_0635	*gltA*	citrate synthase	6.5	8.1e-4
llmg_0636	*citB*	aconitate hydratase	13.9	6.9e-8
llmg_0637	*icd*	isocitrate dehydrogenase	11.3	4.7e-9
E. Amino acid transport and metabolism				
llmg_0362	***dppA***	dipeptide-binding protein precursor	45.3	1.9e-7
llmg_pseudo_09	***dppP***	dipeptide-binding protein	4.6	1.1e-6
llmg_pseudo_42	*leuB*	isocitrate/isopropylmalate dehydrogenase	14.9	1.4e-13
llmg_pseudo_43	*leuA*	2-Isopropylmalate synthase	14.9	3.2e-9
llmg_1284	*leuC*	isopropylmalate isomerase large subunit	13	2.4e-12
llmg_0118	*ctrA*	branched chain amino-acid transporter	7.5	2.4e-6
llmg_pseudo_64	***oppF2***	oligopeptide transport ATP-binding	5.7	1.1e-6
llmg_pseudo_65	***oppD2***	oligopeptide transport ATP-binding	7.5	6.6e-7
llmg_2024	*oppA2*	oligopeptide-binding protein *oppA2*	4	2.4e-6
llmg_2026	*oppB2*	peptide transport system permease	5.3	2.1e-6
llmg_0697	***oppD***	oligopeptide transport ATP-binding	5.3	4.2e-5
llmg_0698	***oppF***	oligopeptide transport ATP-binding	5.6	5.2e-6
llmg_0699	***oppB***	peptide transport system permease *oppB*	5.3	2.2e-5
llmg_0700	***oppC***	oligopeptide transport system permease	5.3	1.9e-5

llmg_0096	*llmg_0096*	glyoxylase	4.9	9.6e-7
llmg_1295	***hisD***	HisD protein	4.9	2.1e-6
llmg_1296	***hisG***	ATP phosphoribosyltransferase	4.6	1.2e-5
llmg_1297	***hisZ***	ATP phosphoribosyltransferase	6.9	1.2e-10
llmg_1298	***hisC***	histidinol-phosphate aminotransferase	8.6	1.2e-10
llmg_1279	*ilvB*	acetolactate synthase catalytic subunit	4.9	1.9e-7
llmg_1280	*ilvD*	dihydroxy-acid dehydratase	4.9	3.6e-8
llmg_1183	*gltB*	glutamate synthase. large subunit	4.3	3.4e-4
llmg_1290	***hisF***	imidazole glycerol phosphate synthase	4.9	1.9e-4
llmg_1291	***hisA***	1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)methylideneamino] imidazole-4-carboxamide isomerase	4.3	1.2e-4
llmg_1278	*ilvH*	acetolactate synthase 3 regulatory subunit	4	1.1e-5
llmg_1452	*llmg_1452*	amino-acid permease	-4	1.2e-4
llmg_1993	*llmg_1993*	transporter	-4.3	1.6e-7
G. Carbohydrate transport and metabolism				
llmg_1873	***glgD***	glucose-1-phosphate adenylyltransferase	8.6	7.2e-6
llmg_1874	***glgC***	glucose-1-phosphate adenylyltransferase	5.7	6.1e-4
llmg_0966[K]	*rmaI*	MarR family transcriptional regulator	5.3	4.1e-4
llmg_0967	*llmg_0967*	permease	8	2.9e-6
llmg_0022	*mtlA*	PTS system mannitol-specific transporter	4.3	2.6e-4
I. Lipid transport and metabolism				
llmg_0154	***cbr***	carbonyl reductase	21.1	1.4e-13
llmg_0155[S]	***llmg_0155***	hypothetical protein	18.4	1.3e-12
llmg_0156[M]	*dltE*	oxidoreductase *dltE*	12.1	9.7e-9
L. Replication, recombination and repair				
llmg_0409	*ssbA*	single-stranded DNA-binding protein	4.3	8.4e-3
O. Post-translational modification, protein turnover, and chaperones				
llmg_0282	*nrdG*	anaerobic ribonucleoside-triphosphate	-4.6	1.1e-4
P. Inorganic ion transport and metabolism				
llmg_1155	*llmg_1155*	Spx-like protein	9.9	3.5e-11
llmg_1138	*mtsA*	manganese ABC transporter substrate binding protein	4.6	3e-4
llmg_0335	***plpA***	D-methionine-binding lipoprotein *plpA*	-5.7	6.1e-6
llmg_0336	***plpB***	D-methionine-binding lipoprotein *plpB*	-4.9	3.6e-7
R. General function prediction only				
llmg_2172	*llmg_2172*	nitroreductase	6.9	3.1e-6
llmg_0095	***llmg_0095***	esterase	6.9	2.7e-7
llmg_0097	***llmg_0097***	flavoprotein oxygenase	4.9	9.3e-7
llmg_0087	*llmg_0087*	short-chain type dehydrogenase	5.3	9.6e-7
llmg_1115	*llmg_1115*	XpaC-like protein	4.3	3.6e-7
S. Function unknown				
llmg_2163[K]	***llmg_2163***	hypothetical protein	18.4	1.4e-13
llmg_2164	***llmg_2164***	hypothetical protein	18.4	2.3e-11
llmg_1659	*llmg_1659*	hypothetical protein	11.3	4.6e-14
llmg_1572	*mycA*	hypothetical protein	5.7	1.5e-8
llmg_0590	*llmg_0590*	hypothetical protein	4.9	2.1e-3

llmg_1263	*llmg_1263*	hypothetical protein	4.3	2.1e-6
llmg_1029	*llmg_1029*	hypothetical protein	4	8.2e-6
T. Signal transduction mechanisms				
llmg_1698	*llmg_1698*	hypothetical protein	4.9	4.6e-5
V. Defense mechanisms				
llmg_1675	***llmg_1675***	ABC transporter ATP-binding protein	9.6	1.9e-5
llmg_1676[M]	***llmg_1676***	ABC transporter permease	8.6	3.4e-4
llmg_0328[X]	*llmg_0328*	hypothetical protein	6.5	3e-4
llmg_0329	*llmg_0329*	ABC transporter ATP binding and	9.6	1.3e-4
X. No predictions				
llmg_0169	*llmg_0169*	hypothetical protein	16	3.6e-8
llmg_1200[E]	***llmg_1200***	hypothetical protein	4.6	2.4e-4
llmg_1201	***llmg_1201***	hypothetical protein	7.5	5.8e-6
llmg_1210[G]	*llmg_1210*	multidrug resistance protein	8	3.9e-11
llmg_1211	*llmg_1211*	hypothetical protein	6.5	1.1e-9
llmg_1283	*llmg_1283*	hypothetical protein	6.1	1.5e-4
llmg_0641	***llmg_0641***	hypothetical protein	5.3	1.6e-8
llmg_0643[P]	***pacL***	cation transporter E1-E2 family ATPase	8	5.9e-7
llmg_1198	*llmg_1198*	hypothetical protein	5.3	2.2e-3
llmg_0985	*llmg_0985*	hypothetical protein	4.9	6.6e-4
llmg_0710	*llmg_0710*	hypothetical protein	-4.6	3.3e-6

[E] Amino acid transport and metabolism; [K] Transcription; [G] Carbohydrate transport and metabolism; [S] Function unknown; [M] Cell wall/membrane/envelope biogenesis; [P] Inorganic ion transport and metabolism; [X] No predictions

Discussion

The capacity of bacterial strains to stabilize emulsions depends on the molecular composition of their cell walls and the resulting surface properties. During the screening of surface properties of 55 *L. lactis* strains it was shown that *L. lactis* cells can be dispersed in water but not in oil (29), (Chapter 3). However, when cells with a high cell surface hydrophobicity (CSH) are mixed with a hydrocarbon, they disappear from the water phase and locate to the oil-water interphase of the emulsion formed. We here observed that the hydrophobic *L. lactis* strains NCDO712, MG1363*pil*, MG1614_clu* all form strong oil-in-water emulsions with petroleum but not with either sunflower, sesame, olive or canola oil. Localisation of cells at the oil-water interphase has been observed previously upon emulsion formation with hydrocarbon (12). The results presented here show that induced cell aggregation improves bacterial emulsification of food-grade oil. The emulsion stability provided by the aggregated bacterial cells is, most

probably, caused by hindering of the coalescence of oil droplets through Pickering stabilization.

The size of oil droplets in buffer, stabilized with bacterial cells, differs significantly between the oil types (data not shown). This phenomenon might be explained either by the variance in the viscosity of the various oils or by the difference in force of hydrophobic affinity of cells to different fatty acids in the oil droplets and in surface tension of the droplets formed during vortexing. When bacterial cells in a food matrix would be seen as colloidal particles one should take into account that bacterial aggregates possess a higher level of organization than other hydrocolloid particles such as proteins or polysaccharides. Another difference exists in the fact that the food (micro) environment not only changes outer surface properties of the cell (50), but also affects the cells' responses (51) and as a consequence thereof their surfaces might change. Additionally, cell surface properties can vary with the growth phase of a cell (29), (Chapter 3). All of these factors clearly distinguish bacteria from inert solid or colloidal particles as emulsion stabilizers.

While we are adding another example of emulsification using bacteria, this study describes for the first time the transcriptional response of microbial cells to residing at an oil-water interphase. A shift in the location of cells from a suspension into an oil-water interphase might alter their cellular metabolism and, possibly, the production of certain flavour compounds. A profound response was evident in the transcriptome of bacteria incubated for 10 min at an oil-water interphase. Especially genes involved in amino acid transport and metabolism were affected. Leucine, isoleucine, glutamate and histidine biosynthesis genes as well as dipeptide and oligopeptide transport genes were up-regulated when cells resided in the emulsion. Interestingly, all of the affected amino acids are essential to *L. lactis* MG1363 (52), a plasmid-cured derivative of the strain NCDO712 used here, as their biosynthesis pathways are not complete. Most of the up-regulated amino acid metabolism-related genes are under control of the global transcriptional regulator CodY (53–55). Highly likely, residing at an oil-water interphase is unfavourable for growth of lactococcal cells, either because nutrients become inaccessible upon localization to the oil droplet surface or because the amount of nutrients is very limited there due to the high density of cells. The elevated expression

of the histidine genes *hisC,Z,D,F,G* and the BCAA genes *leuABC* and *ilvBDH* suggests that *L. lactis* NCDO712 starts experiencing starvation as an earlier report showed evidence of high expression of these genes during starvation (56). Genes for the transport of oligopeptides (the two *opp* operons), of dipeptides (*dppAP*, of which the former is functional, the latter is a pseudogene) and of branched-chain amino acids (*ctrA*, renamed *bcaP* (57)) are all under CodY control (57) and are all significantly up-regulated. This suggests an attempt of the cell to import peptides and/or amino acids as a response to the conditions of starvation. The up-regulated glutamate synthase GltB gene (gene *llmg_1183*) has been shown to be involved in acid stress response (58). High cell densities at the oil-water interphase could potentially lead to high acid concentrations and induce the acid stress. In addition, a number of genes involved in citrate fermentation (*citB, icd, gltA*) were significantly up-regulated, which suggests that pH changes might affect the expression of these genes, despite the fact that strain NCDO712 does not ferment citrate. The *citB, icd, gltA* genes are also under control of CodY, as has been shown for MG1363 (59). Citrate utilisation is strongly pH-dependent (60), and the remainder of the genes present in strain NCDO712 might still respond to the acid stress.

While there seems to be potential for the use of hydrophobic and/or aggregating LAB as clean-label emulsifiers, the amount of bacteria required using the current protocol would prohibit using them for bulk products. Therefore, the amount of cells needed to stabilize an emulsion would need to be reduced to make this a feasible approach. Our results also point out how in a complex environment like a fermented dairy product a heterogeneous cellular response can be brought about by the location of an organism in a particular part of the food matrix. The possibility of selecting starter cultures with altered surface properties was demonstrated recently by conjugating a plasmid from *L. lactis* NCDO712 to a recipient strain that became lactose positive and showed a clumping phenotype (MG1614_clu$^+$) (40), (Chapter 4). Such approaches could be used to steer cells towards an oil-water interphase in a fermented product. This would change its direct environment and, potentially, its metabolic activity. It has previously been shown that flavour profiles could be changed by varying the size of microcolonies

in a cheese matrix (61). The authors speculate that this is due to the localized high cell densities in the colonies leading to altered metabolic activities (61).

There is also an increasing interest in strain selection and screening protocols (49, 62) employing microdroplets of oil or alginate beads in which cells are cultured at high cell densities (63). From the results presented here it is clear that when working with such systems it is important to understand how bacterial cell surface properties might influence the location of a cell within a droplet and how the resulting high cell densities could alter microbial metabolism.

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Supplementary materials for Chapter 6

Table S1. Surface properties of the strains used in this study. PCSH stands for cell surface hydrophobicity with petroleum (%), ST - stationary growth phase; EXP - exponential growth phase, E24 (%) - emulsion stability measured after 24 h in petroleum, ZP (mV) – charge. Number represents average ± standard deviation of three biological replications.

L. lactis strain	Characteristic	Auto-aggregation	Stationary growth phase			Exponential growth phase			Reference
			CSH, %	E24, %	ZP, mV	CSH, %	E24, %	ZP, mV	
MG1363	Plasmid-cured derivative of *L. lactis* NCDO712	no	6±0	0±0	-30±1	7±3	0±0	-39±2	(29)
MG1363*pil*	Ery^R; derivative of MG1363 harbouring pSH74 pilin operon	yes	92±1	85±15	-16±1	96±2	91±14	-13±3	(67)
NCDO712	*L. lactis* dairy isolate, contains the following plasmids: pLP712, pSH71, pSH72, pSH73, pSH74, pNZ712	yes	99±1	100±0	-21±2	99±1	49±3	-20±1	(29)
MG1614 _clu^+	Rif^R, Str^R; Transconjugant, clumping phenotype, derivative of MG1363 harbouring pLP712	yes	90±4	31±4	-36±0	94±1	0±0	-26±0	(40)

Table S2. Numbers of significantly differentially expressed genes in different COG categories. A gene is only represented when its expression level is 4-fold higher or lower ($p < 0.01$) in cells under the two conditions tested: 10 min of incubation at the oil-water interphase in an emulsion or in suspension.

COG function category	No. of genes affected	
	Up-regulation	Down-regulation
C. Energy production and conversion	3	0
E. Amino acid transport and metabolism	26	2
G. Carbohydrate transport and metabolism	6	0
H. Coenzyme transport and metabolism	1	0
I. Lipid transport and metabolism	1	0
K. Transcription	2	0
L. Replication, recombination and repair	1	0
M. Cell wall/membrane/envelope biogenesis	2	0
O. Post-translational modification, protein turnover, and chaperones	0	1
P. Inorganic ion transport and metabolism	8	2
Q. Secondary metabolites biosynthesis, transport, and catabolism	1	0
R. General function prediction only	6	0
S. Function unknown	7	0
T. Signal transduction mechanisms	2	0
V. Defense mechanisms	2	0
X. No predictions	8	1
Total [a]	76	6

[a] Some genes are assigned to more than 1 COG category

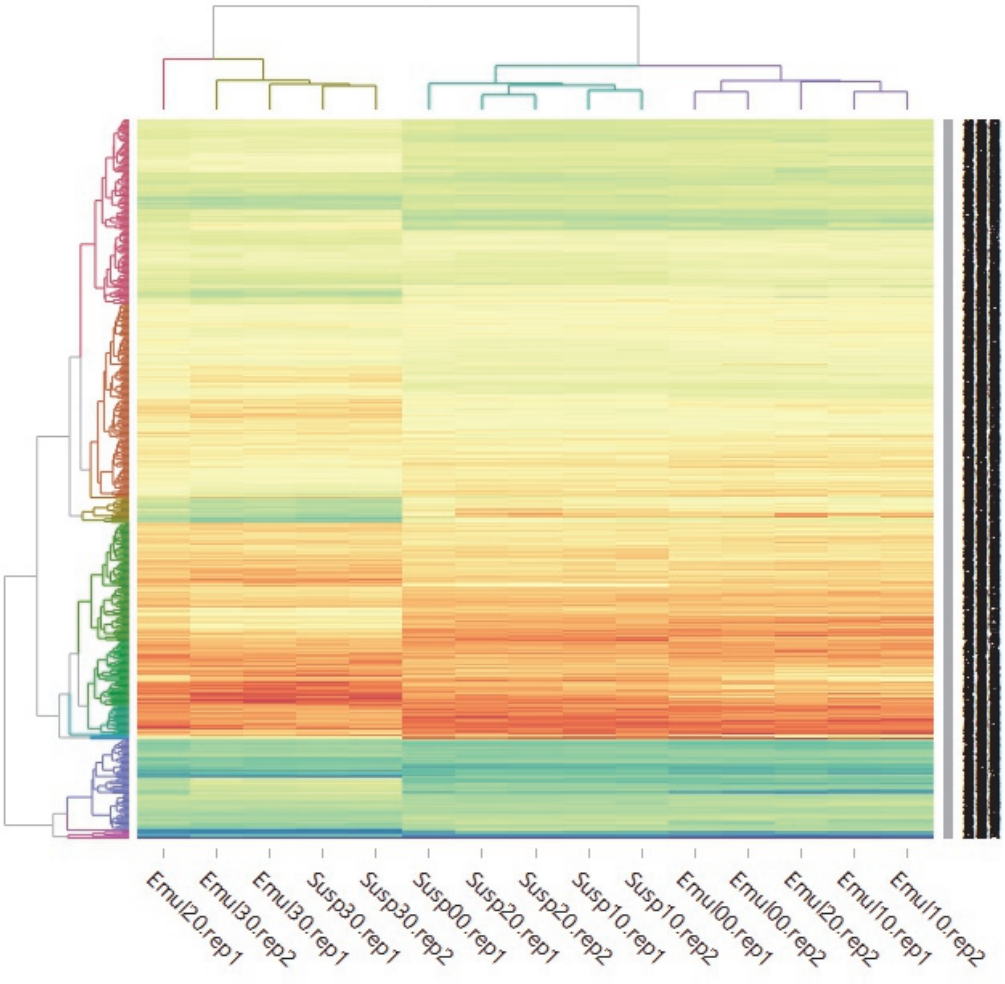

Figure S1. Clustering of RNAseq data based on counts per total counts in million (normalized for total counts per sample) of all expressed genes in *L. lactis* NCDO712. Conditions are labelled by culture medium (emulsion –Emul, suspension – Susp), time of incubation (00 – 0 min, 10 – 10 min, 20 – 20 min, 30 – 30 min), and biological replication (biological replication 1 or 2 - rep1 or rep2). The data of the biological replication 2, cells incubated in suspension for 0 min (Susp00.rep2) was omitted from the analysis due to poor quality of the sample.

Chapter 7

General discussion

Mariya Tarazanova, Herwig Bachmann, Jan Kok

Summary and general discussion

The research described in this thesis aimed at gaining a better understanding of molecular mechanisms involved in interactions between the matrix of fermented dairy products and *Lactococcus lactis,* a Gram-positive bacterial species that is widely used in dairy starter cultures. Cell surface properties are determined by the molecular composition of the cell wall and they play a significant role in interactions between the bacterial cells and the dairy food matrix. The work described in this thesis shows that this holds potential to ultimately influence starter culture functionalities.

Detailed characterization of the dairy isolate *Lactococcus lactis* NCDO712

The interactions between starter cells and dairy product components, as well as the location of the bacterial cells in a dairy matrix are influenced by bacterial surface properties. As it was the aim of this work to get a better understanding of such microbe-matrix interactions, we started out by a detailed characterization of the wild-type dairy strain *L. lactis* subsp. *cremoris* NCDO712 (1). This strain is the ancestor of the most widely used model strain MG1363 (2). The nucleotide sequencing of the entire genome of NCDO712 showed that, next to the chromosome, it contains 6 plasmids (Chapter 2) instead of the 5 reported earlier (1). The additional plasmid, labeled pNZ712, has a size similar to that of the lactose catabolism plasmid pLP712, and this is the most probable reason why it was not detected earlier. Plasmid pNZ712 carries functional nisin immunity (*nisCIP*) and copper resistance (*lcoRSABC*) genes and we could show that the latter can be used as a marker for plasmid transfer through conjugation. Interestingly, *L. lactis* NCDO712 had high surface hydrophobicity, whereas its plasmid-free derivative MG1363 showed very low surface hydrophobicity. This suggests that cell surface hydrophobicity is determined by plasmid-located gene(s). Further, the cell surface-located subtilisin-like serine protease NisP was reported to be involved in cell adhesion to surfaces (3–5). Thus, the pNZ712-encoded NisP might also be a good candidate gene involved in cell surface hydrophobicity. Plasmid pSH74 from NCDO712 was shown to contain a novel pilus gene cluster, which was named *pil*

(Chapter 2). Overexpression of this gene cluster in *L. lactis* strains MG1363, MG1299, and IL1403 resulted in altered cell morphologies, such as cell chaining and fast sedimentation, which were absent in the parental strains. In addition, cell surface hydrophobicity significantly increased, from 5-20% to 95-99%, in the pilus overexpression strains. More specifically, the tip protein SpaC, which takes part in process of cell attachment to a substrate, might be involved in surface hydrophobicity, although this remains to be confirmed. Light centrifugation of cells overexpressing the *pil* gene cluster causes the formation of strong aggregates of which the cells cannot be detached by vortexing. Such strong cell-cell connections might be explained by interactions between the cell surface-located pilin proteins or by interactions between pilin and other cell wall proteins.

In the process of starter culture production, fast sedimentation could make the harvesting of cells more efficient. Besides the effect that pili overexpression has on cell surface properties, it also increases conjugation efficiency. While some of the identified effects of pili overexpression lead to significant phenotypic changes, the actual role of pili in lactococci is still unclear. Oxaran *et al.* described pili in strain IL1403 and showed that deletion of the pilus genes abolished the binding of the mutant lactococcal cells to Caco-2 cells (6). The authors suggest of a role of the pili in the interaction with intestinal cells which is similar to what has been observed in probiotic lactobacilli (7, 8) or with natural a host of *L. lactis,* e.g., udder cells of a cow (9) or with the udder canal cells (10). However, as *L. lactis* did most likely not evolve in the human gut, such attachment is possibly not the primary function of the pili. On the other hand, pili of the plant-associated *Pseudomonas syringae* are involved in the attachment of the bacteria to plant surfaces (11). We did test if overexpression of the pilin cluster increased adhesion to different plant leaves but could not find significant effects (data not shown). While examples for a role in surface attachment may give hints as to the potential functions of the pilus gene cluster identified here, it remains to be determined whether it is an evolutionary remnant from the proposed plant ancestry of *L. lactis* (12) or whether they fulfill a function in the dairy environment.

The regulation of the pilin gene cluster was examined and it was found that the *pil* leader sequence is required for pili expression. Initial attempts to clone the *pil* operon downstream of the nisin-inducible *nisA* promoter or the constitutive *purC* promoter without this leader sequence failed. Three *L. lactis* NZ9000 strains were constructed carrying the pilin gene cluster followed by the gene for green fluorescent protein (GFP) as an easy marker for gene expression. One of the 3 strains contained the leader sequence. However, this strain did not produce GFP at a level significantly higher than that of other two transformants. Fluorescence microscopy showed that all three strains were fluorescent immediately after introduction of the plasmid into the strain but not after that. Growth of the strains in a rich M17 medium with 17% sucrose and 1% glucose resulted in more GFP production than in other media tested (CDM, GM17, whey). Why the native promoter is of importance for successful overexpression of the pilus gene cluster, what the exact role of the leader sequence is in gene expression and how the medium composition exerts its influence are questions that need to be investigated in the future.

Lactococcal cell surface biodiversity and cell surface alteration

To investigate the diversity of lactococcal surface properties, the 55 *L. lactis* isolates of which 25 were isolated from non-dairy (mainly plant) material and 30 from a dairy environment were analyzed (Chapter 3). The cell surface hydrophobicity (CSH), cell surface charge, emulsion stabilizing properties, and the attachment of the bacterial cells to milk proteins were measured. The results demonstrated not only that a large biodiversity in *L. lactis* cell surface properties exists, but also that lactococci isolated from a dairy environment have stronger binding affinity to milk proteins than plant isolates do have. This suggests that protein binding gives an evolutionary advantage to *L. lactis* strains that compete in the dairy environment.

Phenotype-genotype matching allowed identifying 10 candidate genes involved in the modification of the cell surface. Their characterization resulted in the identification of 7 genes that are involved in cell surface properties. It would be interesting to examine whether the alteration (mutation/deletion) of these 7 genes alone or in combinations

would also impact the functionality of the mutant strains in either pure culture and/or in mixed-culture fermentations.

As genetic manipulation to alter cell surface properties is currently not accepted in most jurisdictions, cell surface properties were attempted to change by changing environmental conditions. This was done by e.g., adding ethanol or ammonium sulfate in order to enhance cell aggregation and subsequently cell surface hydrophobicity (Chapter 6). When salt is added to a cell suspension the surface tension of the water increases (13), resulting in the re-arrangement of water molecules, the increase in ionic strength and the Debye-length decrease, as a result of which electrostatic repulsion decreases and hydrophobic interactions increase between cell surface proteins and hydrophobic molecules of a substrate. Interactions between exposed hydrophobic patches of proteins and water molecules are unfavorable. The response of surface proteins is to decrease their exposed surface area in order to minimize contact with water (14). Thus, proteins on bacterial surfaces tend to interact with each other via hydrophobic forces leading to cell-cell contact and consequent cell aggregation and, ultimately cell sedimentation. Such protein-protein contact releases associated water molecules, the entropy of the system increases indicating that these processes are energetically favorable (15). Generally, when ethanol is added, it disrupts hydrogen and hydrophobic bonding of globular proteins, which would usually unfold; the concentration of ethanol needed for protein unfolding varies among proteins (16). After a protein is unfolded, its apolar side chains become exposed to the microenvironment and thus, a protein reacts with other molecules via hydrophobic interactions. We showed that hydrophobic lactococcal strains can form stable oil-in-water emulsions with hydrocarbons in a manner similar to Pickering stabilization, wherein the bacteria act as solid particles that bind to the surface of the oil-water interface and prevent the oil droplets from coalescing. A general rule of Pickering emulsions is that the particle size should be at least 10 times smaller than the targeted emulsion droplet size (17). There is no optimal particle size to make emulsion: it was reported that particles with diameter of about 100 nm can form an emulsion stable for several months with an oil droplet size of 10-30 μm (18), and hydrophilic silica particles

with a primary particle size of ~12 nm form emulsions with droplet size of 2 μm, while aggregated silica particles (size ~150 nm) form emulsions with droplet diameter of about 10-30 μm (19). If it concerns cells as stabilizing particles, then the average emulsion droplet diameter range varies from about 80-100 μm (20) to 500 μm (21). While the formation of bacterial cell-stabilized emulsions worked well with the fluorinated oil HFE7500, it was necessary to induce cell aggregation first with either ethanol or ammonium sulfate to stabilize oil-water emulsions of sunflower seed oil (Chapter 6). This indicated that the emulsifying capacity of cells in oil-water systems is facilitated by solvent-mediated cell aggregation, probably by the ability of the ammonium sulfate to "salt-out" the cell surface proteins and, thus, increasing surface protein hydrophobicity (22). When at this moment mixing by vortexing is applied to cell suspensions containing oil, we hypothesize that protein-oil contacts occur in a matter of seconds via hydrophobic areas in cell surface proteins made available by e.g. the added salt or ethanol. It might also be that the increased cell hydrophobicity is a side effect of cell aggregates. Cell aggregates are better in forming a Pickering emulsion than (single) cells as a consequence of creation a coherent surface layer. Additionally, cell aggregates would increase emulsified oil droplet mass, which might prevent oil droplet creaming, thus, providing longer emulsion stability. All in all, studying microbe-matrix interactions is a challenging task, even on well-characterized *L. lactis* strains like NCDO712 and MG1363. The microbial cell surface composition is complex and strain-specific, which results in a large biodiversity. A complicating factor is the growth phase-dependency of cell surface properties, as we observed in 22 out of 55 *L. lactis* strains, for example in *L. lactis* HP (Chapter 3). The surface hydrophobicity of this strain is >90% in the exponential growth phase and decreases to <20% in the stationary phase of growth. The exact underlying mechanism(s) and which particular surface proteins are involved in hydrophobic interactions of *L. lactis* remain to be identified. In terms of applicability, the enhanced cell aggregation as a result of cell surface hydrophobicity upon salt addition could be used for emulsion stabilization in clean-label or E-number-free applications. Hydrophobic or electrostatic interactions between microbes and matrices can be strain-specific (23), (Chapter 3) due to the specific

biochemical characteristics of cell surface molecular composition (24, 25). To understand the impact of the matrix on lactococcal cells we also studied the transcriptional response of cells residing at an oil-water interphase. The results showed that mostly (46%) genes involved in amino acid and inorganic ion transport and -metabolism were significantly differentially expressed. Of significantly differentially expressed genes 28% have unknown functions; we hypothesize that some of them might be cell surface related and it would be great if additional work would be further conducted in this area.

Functionality of lactococcal cell surface engineering in fermented dairy products

After engineering surface properties and surface morphology of *L. lactis*, it remained to be tested whether these alterations have an impact on the functionality of the bacteria in pure and mixed-culture fermentations (Chapters 4 and 5). The results show that, in fermented milk, gel hardness positively correlated with clumping of cells as a result of pili overexpression, whereas viscosity was higher when milk was fermented with cells with a chaining phenotype or overexpressing pili or exopolysaccharides. It was also observed that the localization of the bacteria in a dairy matrix was dependent on cell surface properties. For example, pili expressing and aggregated cells appeared in tightly filled cavities of the protein matrix of fermented milk while cells that form chains were localized randomly. Clumping, expression of pili, and chaining led to formation of large cell aggregates embedded in the protein matrix in cheese. Furthermore, surface alteration strongly affected the distribution of lactococcal cells in the cheese matrix (Chapter 5). Pili overexpression in *L. lactis* MG1363 and *L. lactis* IL1403 resulted in a significantly increased cell-retention in cheese curd. These results suggest that surface properties of lactococcal dairy starter cultures strongly determine the retention and distribution of the bacteria in dairy food matrices (Fig. 1) and affect textural properties. In this respect, starter culture cells might be considered as structure elements, either by connecting food matrix molecules or as structure breakers or inert fillers in fermented foods, as a consequence of cell surface-dependent properties. The alteration of cell surface properties opens possibilities to modifying starter culture functionality (Fig. 1)

and to use starter cells as structural matrix components. For example, by expressing more charged surface molecules like proteins, glycopolymers or polysaccharides on the bacterial cell walls, the bacteria might function as stabilizers of high viscosity products like puddings, cream cheese, plant-based milk smoothie, dressings, or dysphagia drinks in which electrostatic interactions are the major stabilizing forces (Fig. 1). In this case a product stability might be controlled by the selection of the proper ratio between charged cells and proteins or hydrocolloids used. Possibly, the amount of added salts like e.g acidity regulators like trisodium phosphate and dipotassium phosphates could be lowered in such applications. An alternative way of liquid product stabilization would be via salt bridges when bacterial cells and proteins or when bacterial cells and hydrocolloids are both negatively charged (Fig. 1). To change electrostatic and ion interactions in the food matrix by the alteration of starter cultures still seems to be a challenging task. The simplest way seems to be to screen the strains for surface charge, then group strains based on strong (> -25 mV) or weak surface charge and then create the starter culture mix combining strains with desired surface charge and other properties like fermentation speed or flavour profile. Alternative and more time-consuming way of charge alteration might be a targeted selection of cell surface proteins and the subsequent modification (knock-out, point mutations, or overexpression) of the genes encoding those proteins. In this case it is important to take into account the legislation rules. Incorporation of cells (for example probiotics) in a solid product could be regulated via hydrophobic interactions. To that end it would be important to use probiotic strains with a high surface hydrophobicity e.g. with molecules containing hydrophobic groups or hydrophobic patches like proteins per se or cell surface proteins with high binding specificity for sugar moieties of food polymers: fibers or complex polysaccharides (Fig. 1) that are slowly digestible by a consumer to provide balanced energy after a meal (26). Strong binding of strains to slowly digested fibers or even to proteins would entail a kind of encapsulation of the bacterial cells or its protection against stress conditions. This would allow using such bacteria as probiotics in functional foods. Strain-specific binding to food substrates could even be a beneficial factor in this case as it would allow developing a mixed-strain starter culture with multi-functionality in one application: in fermentation as well as for probiotic purposes.

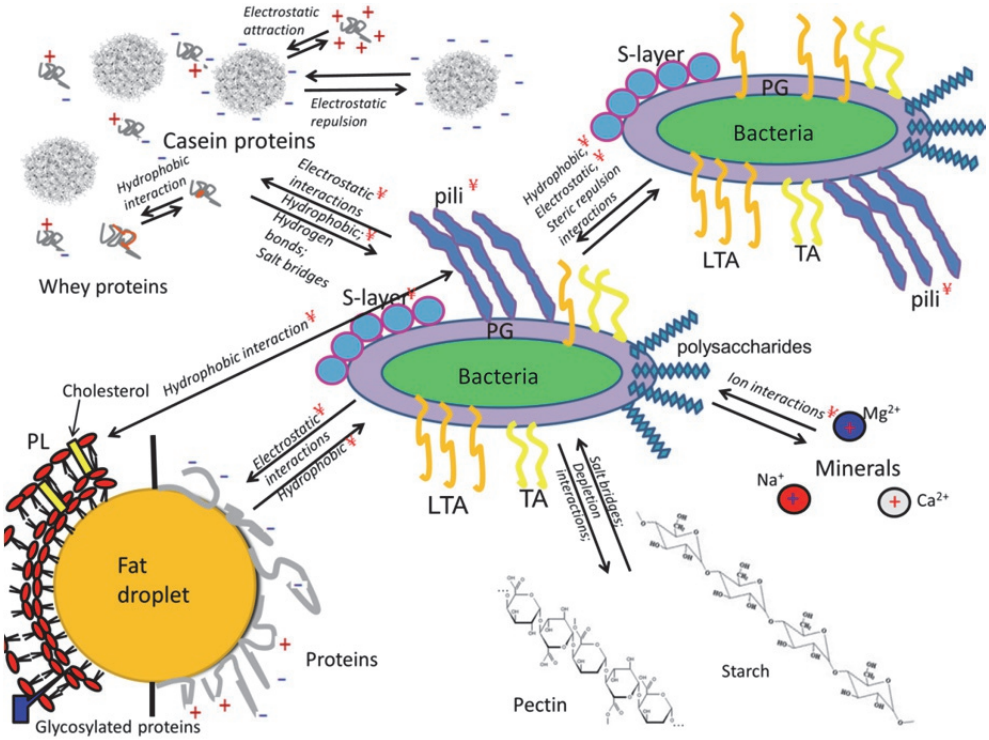

Figure 1. Schematic view of interactions between bacterial cells and components in dairy food matrices such as cheese or fermented milk (not to scale). ¥ Indicates the potential of altering microbe-matrix interactions and subsequently starter culture functionality, as described in this study.

Concluding remarks

Altering cell surface properties and/or cell surface morphology can lead to different starter culture functionality in dairy food matrices. We endeavored to unravel the molecular mechanisms underlying the *L. lactis* surface properties. To employ potential new starter functionalities microbe-matrix interactions need to be studied more intensively as a better understanding thereof is required. One of the challenges that needs to be tackled is that many of the interactions between substrate and bacterial surfaces could be strain specific. Understanding the molecular mechanisms of microbe-substrate interactions should ultimately allow altering bacterial surfaces in the desired direction in a non-GMO way. Using strains with preferred surface properties may result

in improved or even entirely new industrial processes and in enhanced product quality. Up to date genetic techniques are used to obtain bacteria with desired surface properties, in addition to employing chemical components (e.g. alcohols), selective fractionation and enrichment of spontaneous mutants (27). Novel genome sequence data in combination with bioinformatics and comparative genomic analyses hold great promise for the rapid improvement of bacterial industrial functionality (28).

It would be also interesting to study the interactions of surface engineered starter culture cells in combination with hydrocolloids like pectin, carrageenan, cellulose, fiber, and starch which are used as stabilizers of dairy matrices (Fig. 1). The question is whether there are any specific or synergistic effects of microbe-matrix or microbe-microbe interactions that might impact textural properties of an end product like viscosity and/or firmness or lead to a new product matrix? Due to an increasing demand for animal-free products the market of dairy-free products or dairy alternatives is growing, including vegan cheese, vegan spread, dairy-free yoghurt-like desserts, etc. However, taste and texture of the dairy-free alternatives are often still inferior to those of dairy products. The research presented in this thesis paves the way for research into improved or innovative dairy and dairy-free applications and developing new textures and products.

Despite some successful and interesting research results presented here, the knowledge on genes determining starter culture surface properties and microbe-matrix interactions still remains far from complete. Hopefully the questions raised and answers obtained throughout this research will be picked up in future studies.

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Addenda

Samenvatting

Micro-organismen spelen een belangrijke rol in talrijke industriële processen. Melkzuur-bacteriën (LAB) worden bijvoorbeeld wereldwijd gebruikt voor de fermentatie van menselijk en dierlijk voedsel. *Lactococcus lactis* is een van de LAB soorten en wordt gebruikt voor de productie van kaas, kwark en karnemelk, waarin het de smaak, textuur en houdbaarheid van de producten bepaalt. Oppervlakte-eigenschappen van microorganismen worden bepaald door de moleculaire samenstelling van de celwand en beïnvloeden de interaktie van microorganismen met hun omgeving. Deze studie is gericht op het beter begrijpen van moleculaire mechanismen betreffende de interactie tussen *L. lactis* en de matrix van gefermenteerde zuivelproducten.

Om te beginnen werd het genoom gesequencet van de oorspronkelijke zuivelstam *L. lactis* subsp. *cremoris* NCDO712, de voorouder van de veel gebruikte plasmide-vrije laboratoriumstam MG1363. De sequentieresultaten laten zien dat NCDO712 6 plasmiden bevat in plaats van de eerder gerapporteerde 5 plasmiden. Het extra plasmide pNZ712 codeert voor functionele nisine-immuniteit en koper-resistentie. De afmeting van pNZ712 is identiek aan dat van het lactose/protease plasmide pLP712 wat mogelijk verklaart dat het in eerdere studies over het hoofd gezien is. Een van de andere 6 plasmiden, pSH74, bleek een nieuw piline gencluster te dragen. Overexpressie daarvan in verschillende *L. lactis* stammen resulteert in de vorming van haarvormige aanhangsels (pili) op het oppervlak van de cel. Deze op hun beurt leiden tot een hoge hydrofobiciteit van het cel-oppervlak, tot de vorming van ketens van cellen, een snelle sedimentatie van de cellen en reeds bij lichte centrifugatie tot de vorming van sterke aggregaten.

Vervolgens werden de celoppervlaktelading, hydrofobiciteit en emulgeringseigenschappen gekarakteriseerd van 55 *L. Lactis* stammen die geïsoleerd waren uit een zuivel- of bodem/plantenomgeving. Via een nieuw ontwikkelde flowcytometrische methode werd ook de aanhechting van melkeiwitten aan de cel gemeten. De stammen vertoonden een hoge mate van biodiversiteit voor deze

parameters terwijl lactococcen geïsoleerd uit een zuivelomgeving grotere hoeveelheden melkeiwitten bonden dan plantenisolaten. Genotype-fenotype matching van deze 55 stammen resulteerde in kandidaat-genen die betrokken zijn bij celoppervlakmodificatie. Genoverexpressie en –deletie analyses bevestigden de voorspellingen voor wat betreft oppervlaktehydrofobiciteit en melkeiwitbinding van drie van de geïdentificeerde eiwitten terwijl bij sommige van de gemodificeerde bacteriestammen celoppervlakte eigenschappen anders werden beïnvloed dan voorspeld. Na de karakterisering en engineering van *L. lactis* celoppervlakte eigenschappen werden 25 isogene stammen met verschillende kenmerken toegepast in melkfermentatie- en kaasproductie studies. Celoppervlakte eigenschappen konden de textuur van en de ruimtelijke verdeling van cellen in de matrix van gefermenteerde melk beïnvloeden: geaggregeerde cellen vulden de holten in de eiwitmatrix op, terwijl ketens van cellen willekeurig verdeeld leken te liggen. Overexpressie van pili is positief gecorreleerd met gelhardheid en viscositeit van gefermenteerde melk. Een positieve correlatie werd ook gevonden tussen celaggregatie en gelhardheid van gefermenteerde melk. De viscositeit van gefermenteerde melk was hoger wanneer cellen met een keten-fenotype werden gebruikt. Veranderingen in de oppervlakte van *L. lactis* cellen kan ook de celdistributie van de cellen in de kaasmatrix beïnvloeden: klonteren en overexpressie van pili leidden tot de vorming van grote in de eiwitmatrix ingebedde celaggregaten terwijl de expressie van extracellulaire polysachariden (EPS) leidde tot kleine serumgebieden met daarin de cellen. Bovendien bepalen oppervlakte-eigenschappen van de cultuur de verdeling van de cellen over de wrongel en de wei: van de van plasmiden ontdane zuivelstammen bleef 30-72% van de cellen in de wrongel achter, terwijl overexpressie van pili op het oppervlak van cellen de retentie van cellen in de wrongel tot 99% verhoogde. De vorming van ketens van cellen, celaggregatie en EPS-expressie hadden geen invloed op het achterblijven van cellen in kaaswrongel.

De resultaten van beide toepassingsstudies, de productie van gefermenteerde melk en kaas, suggereren dat de functionaliteit van de startercultuur gewijzigd kan worden door de veranderingen aan te brengen in het oppervlak van starterculturecellen. Nieuwe concepten op basis van dit inzicht zullen kunnen leiden tot de ontwikkeling van

gefermenteerde producten met een gewijzigde textuur. Een voorbeeld van van een dergelijk nieuw concept zou de clean-label stabilisatie van olie-in-water emulsies met *L. lactis* stammen via Pickering-stabilisatie mechanisme kunnen zijn. Daarin is de aggregatie van bacteriële cellen van belang omdat die de stabilisatie van oliedruppeltjes verbetert. Hoewel dit conceptueel een interessante benadering is, zou de hoeveelheid cellen die nodig is om een emulsie te stabiliseren, drastisch gereduceerd moeten worden om het haalbaar te maken. Een ander voorbeeld zou het gebruik van startercellen kunnen zijn als structuurelementen zoals ook inerte vulstoffen worden gebruikt, of juist als structuurbrekers in gefermenteerd voedsel.

Deze studie is gedaan om meer inzicht te krijgen in de oppervlakte eigenschappen van cellen van *L. lactis* en hoe die kunnen worden gebruikt om de functionaliteit van de cellen te veranderen. Hoewel de kennis over moleculaire mechanismen die de celoppervlakte eigenschappen bepalen verre van compleet is, en daarmee ook die van de daaruit voortvloeiende microbe-matrix interacties, laat dit onderzoek zien dat er zeker potentie is voor het verbeteren van huidige productieprocessen en voor innovatieve productontwikkeling.

Summary

Microorganisms play an important role in numerous industrial processes. For example, lactic acid bacteria (LAB) are used worldwide in food- and feed fermentations. *Lactococcus lactis* is one of the LAB species used for fermentation of cheese, quark and buttermilk and determines taste, texture, and the shelf life of a product. Surface properties of microorganisms are determined by the molecular composition of the cell wall and influence the interactions of microbes with their environment. This study is focused on a better understanding of molecular mechanisms involved in interactions between *L. lactis* and the matrix of fermented dairy products.

As a starting point, the genome of the wild-type dairy strain *L. lactis* subsp. *cremoris* NCDO712, the ancestor of the widely used plasmid-free model strain MG1363, was sequenced. In contrast to earlier reported evidence, the sequencing results showed that it contains 6 rather than 5 plasmids. The additional plasmid pNZ712 carries functional nisin immunity and copper resistance genes. The size of pNZ712 is similar to that of the lactose/protease plasmid pLP712, which might explain why it has been overlooked previously. One of the other plasmids, pSH74, was shown to contain a novel pilin gene cluster, which when overexpressed in various *L. lactis* strains resulted in appendices on the cell surface. This leads to high cell surface hydrophobicity, cell chaining, fast sedimentation and upon slight centrifugation the formation of strong aggregates.

Next, the cell surface charge, hydrophobicity and emulsification properties of 55 *L. lactis* strains isolated from a dairy or soil/plant environment were characterized. Through a newly developed, flow cytometry-based method the attachment of milk proteins to the cell was measured. A high degree of biodiversity in these parameters was apparent, and dairy lactococci bind higher amounts of milk proteins than plant isolates. Genotype-phenotype matching of these 55 strains resulted in candidate genes involved in cell surface modification. Gene overexpression and deletion analyses confirmed the predictions for three identified proteins involved in surface hydrophobicity and attachment to milk proteins, while in some of the engineered strains cell surface properties other than the predicted ones were affected. After the characterization and

engineering of *L. lactis* cell surface properties, 25 isogenic strains with different characteristics were subjected to milk fermentation and cheese making studies. Cell surface properties altered the textural properties of and spatial distribution of cells in the matrix of fermented milk: aggregated cells tightly fill the cavities of the protein matrix, while chaining cells seemed to be randomly located. Pili overexpression positively correlated with gel hardness and viscosity of the fermented milk. A positive correlation was also found between cell clumping and gel hardness of fermented milk. Viscosity of fermented milk was higher when cells with a chaining phenotype were used. *L. lactis* surface alteration could also change the distribution of cells in the cheese matrix: clumping and pili overexpression led to the formation of large cell aggregates, which were embedded in the protein matrix whereas expression of extracellular polysaccharides (EPS) led to cells residing in small serum regions. Furthermore, the surface properties of the culture determined cell retention in the cheese curd: plasmid-cured dairy strains show 30-72% retention of cells in curd, while pili overexpression on the cell surface increases cell retention to 99%. Cell chaining, clumping or EPS expression did not influence cell retention in cheese curd.

The results of both applications, fermentation of milk and cheese manufacturing, suggest that cell surface alteration should allow modifying starter culture functionality and developing new concepts in formulating fermented products with an altered texture. Another example of a new concept for the application of LAB could be clean label stabilization of oil-in-water emulsions with *L. lactis* strains via the Pickering stabilization mechanism. In this study we have shown that bacterial cell aggregation is of importance in this respect as it improves the stabilization of oil droplets. While this is conceptually an interesting approach, the amount of cells required to stabilize an emulsion would need to be reduced considerably to make this a feasible approach. In yet another example starter cells could be employed as structure elements such as inert fillers or as structure breakers in fermented food.

This work was aimed at getting more insight in the surface properties of *L. lactis* and how these could be used to alter the functionality of the cells. While the knowledge of the molecular mechanisms determining *L. lactis* cell surface properties and,

subsequently, the microbe-matrix interactions are far from complete, this work and future research on the microbe-matrix interactions certainly holds a potential for improving current manufacturing processes and developing novel products.

Резюме

Микроорганизмы выполняют важную роль в различных индустриальных процессах. Например, молочнокислые бактерии используются по всему миру в производстве пищевых продуктов питания и кормов. *Lactococcus lactis* является одним из видов молочнокислые бактерий, используемых для изготовления сыра и сквашивания молока. *L. lactis* формируют вкус и текстуру конечного продукта и определяют срок его хранения. Свойства поверхности микроорганизмов (заряд, гидрофобность, эмульгирующая способность) определяются молекулярной композицией клеточной стенки микроорганизмов. Поверхностные свойства клеточной стенки влияют на взаимодействия микроорганизмов с окружающей их средой. В данном исследовании основное внимание уделяется углубленному пониманию молекулярных механизмов, вовлеченных во взаимодействия между *L. lactis* и матриксом сферментированного молочного продукта.

В качестве отправной точки был секвенирован молочный штамм дикого типа *L. lactis* subsp. *cremoris* NCDO712, являющийся предком широко используемого в лабораториях штамма MG1363, который не содержит ни одного плазмида. В отличие от ранее сообщенных данных, что NCDO712 содержит 5 плазмид, результаты секвенирования показали наличие 6 плазмид. В данном исследовании показано, что дополнительный плазмид pNZ712 содержит работающие гены, отвечающие за иммунитет к низину и меди. Размер этого плазмида аналогичен размеру плазмида pLP712, несущего функции утилизации лактозы и молочных белков. Этот факт может быть причиной, по которой pNZ712 не был обнаружен в более ранних исследованиях с использованием детекции размера плазмида на геле. Также в этом исследовании показано, что еще один плазмид, pSH74, содержит новый, до этого не идентифицированный кластер генов, кодирующих поверхностные белки - пили. При их сверхэкспрессии в *L. lactis* MG1363, MG1299, IL1403 на поверхности клеточной стенки этих бактерий образуются белки нитевидной формы, которые придают

высокую гидрофобность клеточной поверхности, а также формирование клеточных цепочек, быстрое осаждение микроорганизмов, и при небольшом центрифугировании - сильное образование агрегатов клеток, в то время как родительские штаммы не демонстрировали этих фенотипов.

Затем были охарактеризованы свойства клеточной поверхности у 55 *L. Lactis* штаммов, выделенных из молочной или почвенной/растительной среды. На основе проточной цитометрии был разработан метод, который позволил определить процент присоединения белков молока к исследуемым бактериям. Данные показали высокую степень биоразнообразия поверхностных свойст бактерий, а лактококки, выделенные из молочной среды, присоединяли большее количество белков молока по сравнению с растительными изолятами. Это может быть объяснено выборочным преимуществом для бактерий выделенных из молочной среды, которые обычно имеют больше аминокислотных ауксотрофов по сравнению с бактериями из растительной среды обитания. Сопоставление генотипа-фенотипа этих 55 штаммов позволило предсказать гены, участвующих в модификации клеточной поверхности. Анализ сверхэкспрессии и делеции генов подтвердили прогноз для трех идентифицированных белков, участвующих в поверхностной гидрофобности бактерии и в прикреплении к молочным белкам, хотя некоторые из наших сконструированных штаммов показали измененные свойства поверхности клеток, отличные от прогнозируемых. После характеристики и конструирования свойств поверхности клеток, 25 изогенных штаммов *L. lactis* с различными свойставами клеточной поверхности, били применены в сбраживании молока и в производстве сыра. Результаты показали, что свойства клеточной поверхности изменили текстуру и пространственное распределение клеток в матриксе сферментированного продукта: бактерии, формирующие агрегаты, плотно заполняли полости белкового матрикса, а бактерии, формирующие цепочки, по-видимому, расположились случайным образом. Сверхэкспрессия пили положительно коррелировала с твердостью геля и вязкостью сброженного молока. Положительная корреляция была также

обнаружена между клетками, формирующими агрегаты, и твердостью геля сферментированного молока. Вязкость сферментированного молока была выше при использовании бактерий, формирующих цепочки. Конструирование поверхности *L. lactis* повлияло на распределение клеток в матриксе сыра: бактерии склонные к формированию клеточных комков и сверхэкспрессия пили привели к формированию крупных клеточных агрегатов, которые были плотно включены в протеиновый матрикс сыра, тогда как экспрессия экзополисахаридов привело к формированию небольших областей сыворотки в сыре, в которых и находились эти бактерии. Кроме того, поверхностные свойства заквасочной культуры определяют процент удержания клеток в сыре в процессе его изготовления: безплазмидные молочные штаммы показали 30-72% удержания клеток в сыре, в то время как сверхэкспрессия пили на поверхности клеток увеличила удержание клеток в сыре до 99%. Штаммы, формирующие клеточные цепоки и комки, а также экспрессия экзополисахаридов не повлияли на удержание клеток в сыре.

Результаты ферментирования молока и производства сыра свидетельствуют о том, что изменение поверхности клетки позволит модифицировать функциональность заквасочных культур и, таким образом, разработать новые или улучшенные ферментированные продукты с измененной текстурой. Другим примером новой концепции является применения молочнокислых бактерий, таких как, например, *L. lactis*, в качестве стабилизатора масло-в-воде эмульсий методом Пикеринга, что позволит использование «чистой этикетки» при маркировке. В этом случае агрегация бактериальных клеток имеет важное значение, поскольку улучшает стабилизацию масляных капелек. Хотя это концептуально интересный подход, количество бактерий, необходимых для стабилизации эмульсии, нужно оптимально подобрать, чтобы сделать этот подход приемлемым. Еще одним примером может быть использование заквасочных культур в качестве структурных элементов, таких как инертные наполнители или в качестве структурных разрушителей в ферментированной пище.

Эта работа была направлена на то, чтобы получить более полное представление о поверхностных свойствах *L. lactis* и о том, как свойства клеточной поверхности можно использовать для изменения их функциональности. В то время как знания о молекулярных механизмах, определяющих свойства поверхности клетки *L. lactis*, и последующие взаимодействия между бактериями и матриксом продукций далеки от завершения, эта работа и дальнейшие исследования безусловно обладают потенциалом для улучшения текущих производственных процессов и разработки новых продуктов.

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About the author

Mariya Tarazanova (1988) was born in Moscow, Russia. In 2004 she entered the Russian State Agricultural Univesty (RSAU-MTAA) to study in the MSc Food Technology program. In 2008 Mariya took part in an Erasmus program which brought her for a half year to Wageningen University (specialization: Product Functionality) in the Netherlands. After getting a bit of insight on the education system and successfully finishing the required courses she returned back to Moscow to obtain her MSc degree in 2009. At the same time Mariya was applying for a Dairy Science and Technology fellowship and education of Food Technology program and eventually was selected for this study. In 2009 Mariya came back to Wageningen University to complete her MSc Food Technology education under supervision of Kasper Hettinga and Hein van Valenberg. During her MSc education, Mariya did some research on adsorption characteristics of different phospholipid species on fat globule (Danone) as well as on proteolytic activity in raw milk rated to somatic cells (WUR in collaboration with NIZO). In 2011 she obtained her second MSc degree. Meanwhile the PhD project on Microbe-Matrix interaction in dairy starter culture functionality was announced within the Top Institute Food and Nutrition in collaboration with NIZO and RUG. After graduation, Mariya was very delighted and fantastically happy to start working on this subject which combines the area of dairy science and microbiology/molecular genetics. The work was carried out at NIZO under supervision of Dr. Herwig Bachmann and Prof. dr. Jan Kok. The project was aimed at better understanding the molecular mechanisms involved in the surface properties of the industrially important lactic acid bacterium *Lactococcus lactis* and how the molecular mechanisms could be used to alter the functionality of the cells in industrial food fermentation (namely dairy) processes. The results of this project are described in this thesis and are published in scientific journals. Since January 2016 Mariya works as specialist nutrition and texturizing at the Application Development Center of Avebe.

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